

The bioavailability of Selenium enriched milk proteins

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Introduction

Due to the relatively low level of selenium (Se) in Australian and New Zealand soils many primary food products from these countries have relatively low Se levels (Combs. 2001). While it is generally accepted that Se intakes of Australian and New Zealand consumers are sufficient to ensure no overt signs of deficiency, there is a feeling that the relatively low intakes may contribute to elevated risk for some cancers (eg. bowel) (Clark *et al.* 1996). However, Se supplementation is problematic, as high Se intake can be toxic, particularly if the source is inorganic (Ortman *et al.* 1999). Protein-bound Se is more bioactive and less toxic than inorganic forms of Se and there is interest in delivering Se in organic forms in food products we consume.

Aims

The overall objective of the project was to increase the competitiveness of the dairy industry in Victoria, Australia, by identifying and confirming the benefits of Se rich milk proteins to human health, using the pig as a model. This is assuming that milk protein products are a more effective delivery route of Se than grain or meat for dosing human at 'supranutritional' levels. The aim of the study was to determine the relative bioavailabilities of Se from the Se-enriched whole milk and milk replacers supplemented with exogenous selenate and Sel-Plex® (Alltech Biotechnology) in

neonatal pigs.

Importance of Selenium

The first indication of the nutritional importance of selenium appeared in the 1950s; Schwarz and Foltz (1957) identified selenium as "essential to animal health" when they discovered that trace amounts protected against liver necrosis in vitamin E-deficient rats. In the early 1970s, experiments (Rotruck *et al.* 1972) revealed selenium as an essential component of glutathione peroxidase, which is an important enzyme for the process that protects polyunsaturated membrane lipids from oxidative degradation.

Today Se is regarded as an essential micronutrient in the diet of many life forms, including humans and other mammals (Berlau *et al.* 2004). The beneficial role of selenium has been studied worldwide and it is rapidly becoming recognized as one of the more promising cancer chemo-preventative agents. The breakthrough discovery between supra-nutritional doses of Se and possible cancer protection came from Clark *et al.* (1996), and has resulted in considerable research to elucidate fully its mode of action. With 40 years of research, epidemiological studies using *in vivo* models have shown that a dietary intake of Se reduces cancer incidence in a variety of animal models (Meulliet *et al.* 2004).

The level at which Se is considered likely

to be most effective in chemo-prevention exceeds that in daily recommended allowances 50-70µg/day (approximately 70µg in men and 50µg in women), with 40µg/day as a minimum requirement (National Academy of Sciences). However, 100-200µg Se/day has been shown to inhibit cancer development in humans, with 400µg/Se/day being considered the upper limit (El Bayoumy, 2001).

In addition to cancer, there is evidence to suggest that Se also has a role in: inhibiting viral expression, slowing the ageing process, delaying the, preventing heart disease and other cardiovascular diseases and mammalian development. Se is also required for the proper functioning of the immune system, and appears to be a key nutrient in counteracting the development, virulence and progression of AIDs in HIV-infected patients (Rayman 2000). The range of biological processes, which are potentially dependant on optimal selenoprotein and selenoperoxidase activity in mammals, emphasises the importance of achieving adequate selenium intake in the diet.

Selenium deficiencies Se deficiency in animals has long been recognised (since the 1950s), although obvious clinical signs of human Se deficiency are rare. The exception comes from the discovery of juvenile cardiomyopathy known as Keshan disease in the early 1930s in China. Investigations into the epidemiology of Keshan disease revealed that individuals living in areas with Se-poor soils were susceptible to the disease, hence the disease is likely to involve a nutritional deficiency of the essential trace mineral Se (Ishihara *et al.* 1999). The role of nutrition in infectious disease has long been associated with changes in the immune response of the nutritionally deficient host. It has been shown in a number of studies that nutritionally deficient humans or animals are more susceptible to a wide range of

infections. This increase in susceptibility is thought to be the result of an impaired host immune response to a deficient diet. However, recent studies have demonstrated that not only is the host immune response affected by the deficient diet, but the viral pathogen itself can also be altered (Beck and Levander. 1998). Dietary deficiencies (Se) that lead to oxidative stress (possibly due to the biological role of Se in the form of GPx) in the host can alter a viral genome such that a normally benign form of mildly pathogenic virus becomes highly virulent in the deficient, oxidatively stressed host (Scrimshaw. 1975). Once the viral mutations occur, even hosts with normal nutrition can be affected by the newly pathogenic strain.

Selenium enriched animal products

The recent suggestion that Se may have protective qualities in humans, raises the interest in improving the Se status of human beings in Se-deficient areas. From numerous studies it is clear that the supplementation of the diet of dairy cows with organic Se may be one way of increasing the Se intake of animals and humans. Combinations of foods such as dairy foods with cereals and pro-biotic bacteria with increased Se content provide potentially interesting alliances in reducing cancer and other disease risks.

It is clear that there needs to be a vector for transporting Se into the human diet. The versatility of milk suggests milk is an ideal vehicle for producing Se-rich proteins, as a terminal pool it is feasible to enhance the concentrations of Se in the milk with ease. It has already been demonstrated that it is possible to produce milk protein products such as MPC80 and casein powders, which contain 3-4.9, and 2.4-6.6ppm, respectively. At these concentrations, these can be considered to be nutraceuticals suitable for prophylactic treatment of humans considered to be at risk from developing diseases, such as

cancer of the colon (Rayman, 2000). Further experimental evidence is accumulating from animal models and *in vitro* data, which show that dietary proteins can influence cancer expression. Dairy proteins in particular have been shown to be relatively protective when compared with defatted soybean and red meat in the dimethyl hydrazine-induced (DMH) colon cancer model.

Selenium Absorption

Several forms of selenium enter the body as part of amino acids within proteins. The two most common forms of the element that enter the body are Se-met and Se-cys which are found mainly in plants and animals respectively. The primary sites of absorption are from throughout the duodenum. The greatest absorption of Se-met is absorbed from the duodenum, followed in decreasing amounts by the jejunum and ileum, with virtually no absorption occurring in the stomach. (Combs and Combs. 1986). However, absorption of inorganic forms of the element varies widely due to luminal factors, this variation in absorption reduces total absorption of all forms. Se absorption is not affected by body selenium status, the absorption of Se is closely related to multiple nutritional factors that inhibit or promote absorption. Vitamins A, C, and E along with reduced glutathione enhance absorption of the element. In contrast, heavy metals (i.e. mercury) decrease absorption via precipitation and chelation (Combs and Combs. 1986).

Methods: Animals and handling

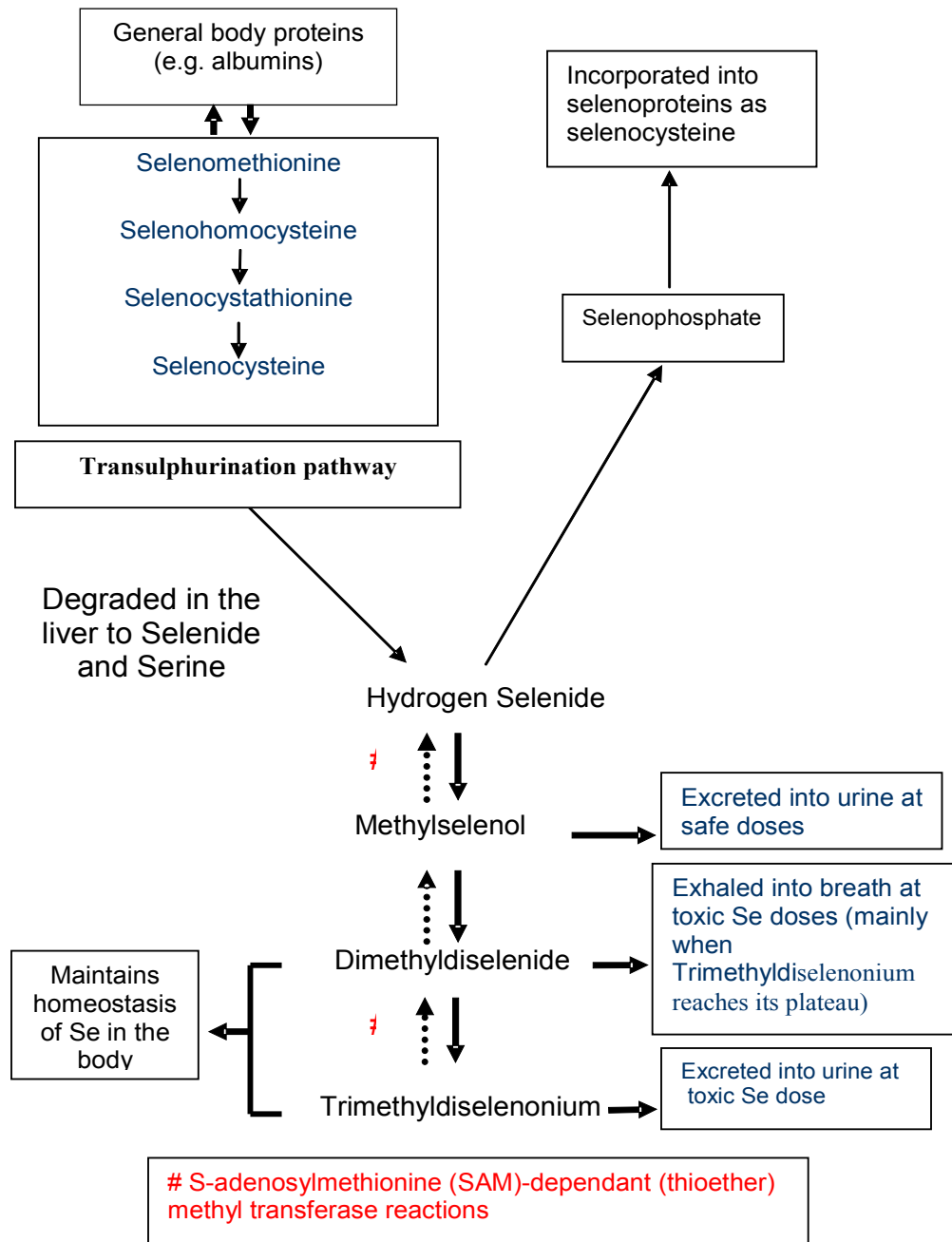
All procedures involving animals were approved by the Department of Primary Industries VIC Government Australia Ethics Committee. Milk to produce the enriched Whole Protein Concentrate 80 (WPC80) was obtained from cows fed supplemental diet containing SelPlex® (organic form of selenium from yeast). The diets used in the study are outlined in Table 1. Diet 1 contained milk

enriched with Se (1070µg Se/kg DM) obtained from cows fed a supplemented diet containing SelPlex®, whilst the control diet (diet 2) (135µg Se/kg DM) was made using milk powder from non-supplemented cows. Additional diets (diets 3 and 4) were formulated by adding either selenate (diet 3) or SelPlex® (diet 4) to the control non-supplemented diet, to give Se concentrations of 1070µg Se/kg.

The study involved 72 male crossbred (Large White x Landrace) weaner pigs at 2 days of age, taken once they had received colostrum from the sow, been injected with 100mg of Fe, and had their teeth clipped. They were then trained to drink milk from a teat in approximately 3 days. The aim was to have 3 replicates on each diet at each age, only a maximum of 14 piglets were taken at any one time to ensure that they received the correct amount of care and attention (DPI Ethics Committee). The piglets were transferred from the farrowing shed to the weaning shed and put in to individual wire mesh cages (0.6m x 0.45m x 0.5m high). The cages were located in an insulated building in which the ambient temperature was maintained between 26 and 32°C, with additional heat provided by a 175W infra-red lamp (see picture below).

In the first 2-4 days the piglets were fed 3 times a day, once in the morning (6am-8am), in the afternoon (12am-2pm) and in the evening (5pm-7pm). They were all hand fed with approximately 100-150g of whole milk powder, with an added supplementation (5ml/500g⁻¹) of probiotics, *Lactobacillus casei Shirota* found in Yakult. One of the real problems with liquid feeding is that hygiene is paramount, and even when precautions are taken, enterotoxigenic bacterial invasion can occur. Supplementation with the fermented milk drink Yakult also aimed to reduce the risks of enteric infection through assisting in maturation

Fig 1: Metabolism of Selenium in the human (modified: Combs and Combs. 1986, Combs and Lu. 2001 and Rayman. 2001).



of the natural micro flora in the gut (Dunshea *et al.* 1999). This is important as the change from the sow feeding every 45mins letting down only 20mls of milk per feed, to fewer feeds and more volume (5-10 times more), makes the piglets digestive system hyper sensitive, especially to the *E. coli* bacteria.

All of the piglets were randomly allocated one of 4 diets and slaughter days (0, 7, 14, 28, 42 days) after 3-4 days once they had been trained to feed from the teats, or when they had reached the same weight as when taken from the sows. Once on their diets they were fed twice a day and drank *ad libitum* from 1.75L bottles attached to the side of the cages, refusals were also recorded.

The bottles were washed with dairy detergent and then rinsed with bleach to minimize risks of any bacteria growing in the bottles. The dietary treatments were designed to be adequate for all nutrient requirements and to ensure a growth rate of approximately 150 - 200g per day, pigs were weighed twice per week and their feed adjusted to 1.7MJ/kg body weight to maintain growth rates.

Blood, tissue and hair sample collection

The piglets were restrained on a wooden V-board to obtain a blood sample (approximately 8mL) from the jugular vein by venipuncture into lithium heparin vacutainers (Grenier bio-one, Kremunster, Australia). A hair sample was taken simply by shaving a small patch, preferably in the middle of the piglet, so as to avoid contamination from milk around the mouth, or faeces, which may contain excess Se. Samples of blood and hair were taken before the piglets started on the specified diets at 4 days, and then on 7, 14, 28 and 42 days thereafter. This method was approved by the Department of Primary Industries Animal Ethics Committee. Plasma samples were placed on ice immediately

then separated and snap frozen to be later analyzed for Se concentration.

Se concentration was calculated using a Vapour Generation Accessory-Inductively Coupled Plasma-Mass Spectrometry (ICP-MS), which is a very powerful tool for detecting and analyzing trace and ultra-trace elements such as selenium.

The piglets (three per diet group), were euthanized at 7, 14, 28 and 42 days of age, via an intravenous injection of sodium pentobarbitone. An additional 4 pigs were slaughtered at 4 days of age, after accumulation of the control diet and after they had returned to approximate weaning weight, to provide an initial tissue selenium content and other biomarker data. Various tissues were obtained from the pigs including; muscle, fat, liver, kidney, hoof, brain, thymus and colon (both proximal and distal), these were snap frozen in liquid nitrogen and stored at -80°C to be later analysed for Se concentrations and the expression of established biomarkers of Se (for example GPx, Selenoprotein-P).

Results: Gpx activity

Cellular glutathione peroxidase (c-GPx) is a member of a family of GPx enzymes whose function is to detoxify peroxides in the cell. Because peroxides can decompose to form highly reactive radicals, the GPx enzymes play a critical role in protecting the cell from free radical damage, particularly lipid peroxidation (Arthur *et al.* 1997). The GPx enzymes catalyze the reduction of Hydrogen Peroxide (H_2O_2) to water and organic peroxides (R-O-O-H) to the corresponding stable alcohols (R-O-H) using glutathione (GSH) as a source of reducing equivalents (see fig 3).

With the exception of phospholipid-hydroperoxide GPx, a monomer, all of the GPx enzymes are comprised of 4 identical subunits (monomer Mr 22-23 kDa) (Epp *et al.* 1983). Each subunit contains a

Ingredients (g)	Diet 1	Diet 2	Diet 3	Diet 4
High Se Liquid Milk	5000.0			
Water		4325.0	4325.0	4325.0
Whole Milk Powder		675.0	675.0	675.0
SelPlex® 135µg/g (g)				5.0
Sodium Selenate 27µg/mL (mL)			25.0	
SOLUVET Liquid Vitamins (mL)	1.5	1.5	1.5	1.5
TRACEMIN Liquid Trace Min (mL)	15.0	15.0	15.0	15.0
IRON DEXTRAN (mL)	1.0	1.0	1.0	1.0

Table 1: ingredients to make 5kg of diets



Fig 2: Project environment for piglets

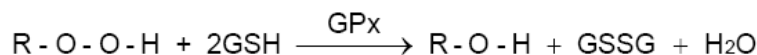


Fig 3: Reduction of Hydrogen Peroxide by GPx (modified Epp *et al.* 1983).

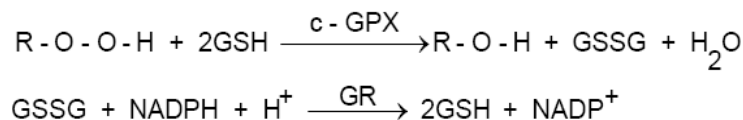


Fig 4: Oxidation of glutathione, reduction of organic peroxide by c-GPx (modified Epp *et al.* 1983).

molecule of selenocysteine in the enzyme active site. The selenocysteine is thought to participate directly in electron donation to the peroxide substrate and become oxidized in the process. The enzyme then uses glutathione as an electron donor to regenerate the reduced form of the selenocysteine (Arthur *et al.* 1997).

The GPx-340nm assay is an indirect measure of the activity of c-GPx. Oxidized glutathione (GSSG), produced upon reduction of an organic peroxide by GPx, is recycled to its reduced state by the enzyme glutathione reductase (GR) (see fig 4)

The oxidation of NADPH to NADP⁺ is accompanied by a decrease in absorbance at 340 nm (A 340nm), thus providing a spectrophotometric means for monitoring GPx enzyme activity. To assay GPx, a cell or tissue homogenate is added to a solution containing glutathione, glutathione reductase, and NADPH. The enzyme reaction is initiated by adding the substrate, tert-butyl hydroperoxide and the A 340nm is recorded. The rate of decrease in the A 340nm is directly proportional to the GPx activity in the sample.

Results: Analysis of data

All statistical analyses were carried out using GenStat®. One-way analysis of variance (ANOVA) was performed to determine if varying the source of Se

supplementation caused a significant difference in plasma Se concentration, or GPx activity.

A Generalized Linear Model ANOVA, and a Generalized Quadratic Model ANOVA were performed to determine if there were any significant differences caused by the Differences in diet and age on plasma Se concentration and GPx activity.

With the use of Microsoft Excel software tables were drawn up and graphs were plotted depicting the statistical data obtained.

Results: Discussion

The results of the ANOVA of plasma GPx showed that there is a significant difference, and a linear relationship between diet and age, where $P \leq 0.001$. These results are supported by liver GPx activity ANOVA, with a significant difference between diet and GPx activity, with a P value of 0.021 ($P \leq 0.05$). Due to the significant results graphs can be drawn (see fig 5 and 6) thus showing the linear relationships between GPx activity, diet and age. The graphs depicting the linear relationship between GPx activity, diet and age can be seen in figure 5 and 6. The activity of Gpx differs depending on the amount of Se present in the diet. The lower the absorption at 340nm (A 340nm) the greater the GPx activity and it assumed more highly bioavailable the source of Se.

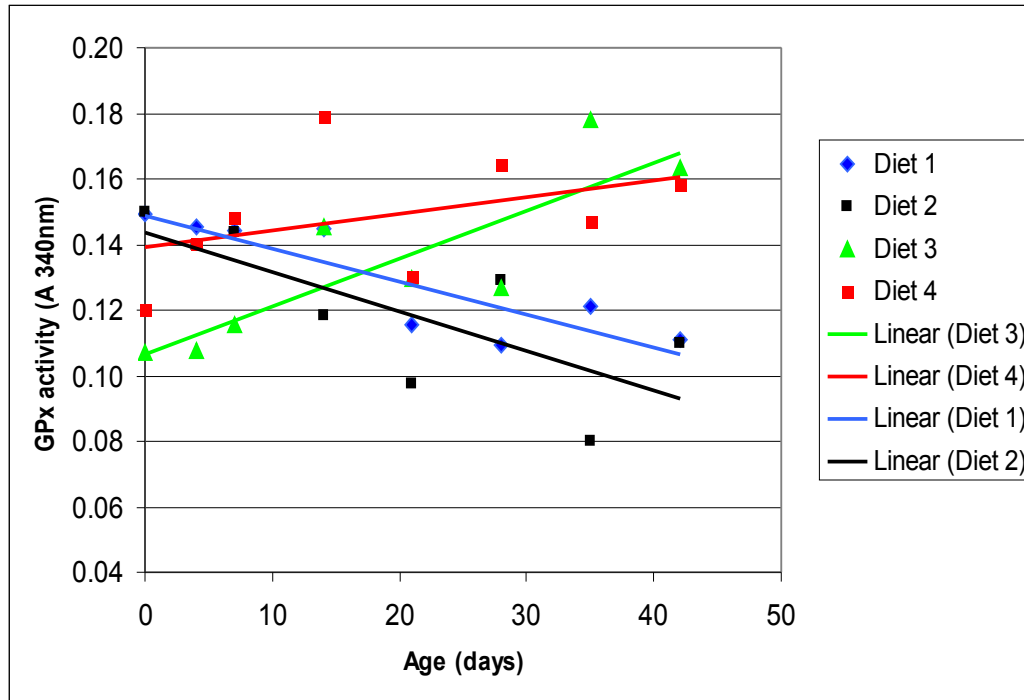


Fig 5: linear relationship of GPx activity at 340nm absorbance of all diets (HSeM, LSeM, HSN, HSP) and at all ages (0, 4, 7, 14, 21, 28, 35 days) in blood plasma.

Diet 1 = HSeM (milk from cows fed supplemental diet containing SelPlex®), Diet 2 = LSeM (Control = whole milk powder), Diet 3 = HSN (Control + Selenate) Diet 4 = HSP (Control + SelPlex®)

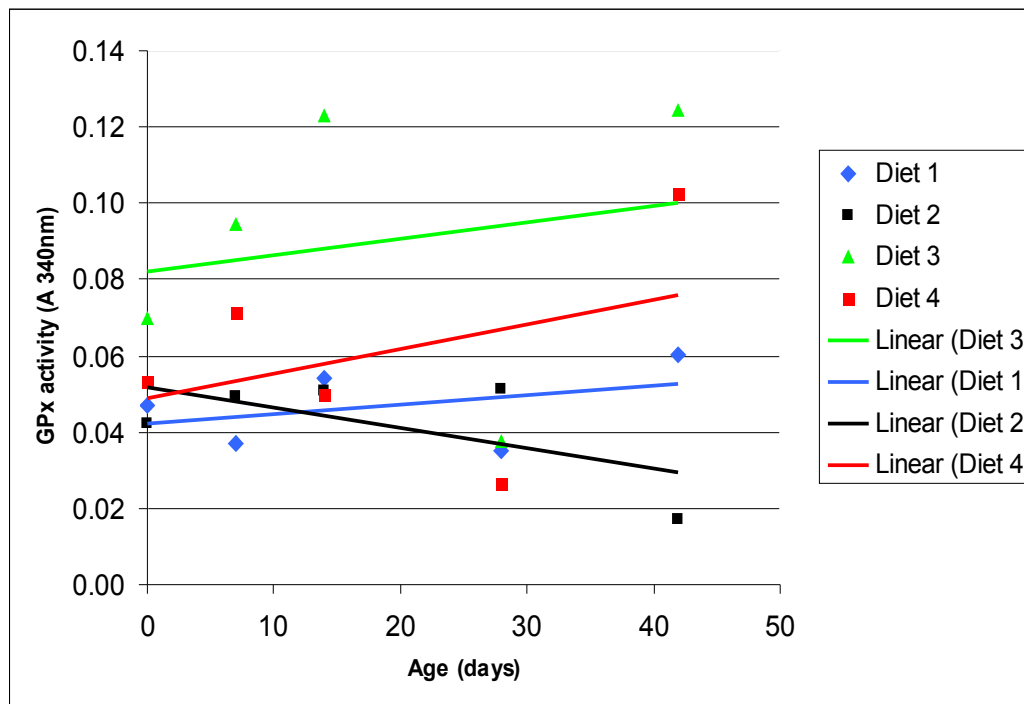


Fig 6: linear relationship of GPx activity at 340nm absorbance of all diets (HSeM, LSeM, HSN, HSP) and at all ages (0, 4, 7, 14, 21, 28, 35 days) in liver samples.

Increasing attention is paid world-wide to the comparison of effects of organic and inorganic selenium. In the present study there are two organic forms of Se fed to the neonatal piglets, protein – bound, diet 1, and SelPlex®, diet 3, there was also an inorganic form of Se supplementation, selenate, diet 3. Results showed the largest increase in plasma selenium concentrations from 93.5 µg.l⁻¹ to 147.2 µg.l⁻¹ when supplementing with organic Se from protein – bound Se, results from inorganic Se supplementation showed little change. The same results were reported by Ortman and Pehrson (1997) who arranged a similar experiment and measured selenium concentrations in whole blood, blood plasma, and milk of dairy cows.

Selenium sources have quite variable responses, but mainly when comparing organic and inorganic sources. Inorganic Se (selenate or selenite) is currently only approved for incorporation into livestock diets in certain countries. In the present study, at identical dietary concentrations diet 3 (selenate) and diet 4 (SelPlex®) had similar biological responses and GPx activity, and only a slight increase in Se plasma concentration in diet 3 compared with diet 4, but more efficiently utilized compared to the control diet. This does not support the findings by Mahan *et al* (1975), which suggested that an organic form of Se is better retained. However when looking at the results of diet 1, a protein – bound, organic source of Se, there is strong evidence that diet 1 is more biologically available compared with the other diets.

Two theories; conflicting minerals or vitamins and the mechanism of intestinal; absorption, offer explanations as to why absorption rate and therefore bioaccumulation of Se in the body varies, and thus why there will be different results of plasma Se concentration and GPx activity. Two macrominerals, calcium (Ca) and sulphate (S), have been shown to

influence Se utilization. Maximum selenium absorption occurred when diets contained 15 g Ca/kg. Considering this information, there may have been an influence of the Ca in the diets on Se status in the pigs. The approximate concentration of Ca in the diets was only 3 g Ca/kg which is well below the suggested optimum of 15 g Ca/kg, therefore may have affected Se absorption and utilization. Elevated intake of sulphate (from water or feed) for long periods of time also appears to reduce selenium status of animals fed selenate. S reduces uptake of selenium (from selenite), this changes the form of Se reaching the intestine and thus reduces its absorption. It is also thought to interfere with the intestinal selenite transport system. It is unlikely that S had a major influence over digestion of Se in the diets, but it does provide evidence that Se may be changed in to less digestible forms.

The predominant form of Se in Se-yeast contained in SelPlex® is selenomethionine (Se-met). Digestion is completely different for inorganic and Se-met; therefore, factors that reduce absorption of inorganic Se are unlikely to influence absorption of Se-met. The metabolism of inorganic Se and Se-met within a cell also differs. Inorganic Se is used almost exclusively in the synthesis of selenospecific enzymes; whereas, Se-met can be used in the synthesis of those enzymes, but it can also be incorporated into any protein that contains methionine. A study conducted in 1998 suggested that the organic forms of selenium increased blood selenium concentration to a greater extent than inorganic forms. However, it did not significantly improve the activity of the selenium-dependent enzyme, glutathione peroxidase (Combs and Gray. 1998). Data on the true digestibility of Se from Se-met or Se-yeast are very limited and variable.

The results of the present experiment have extended the current knowledge on effects of oral administration of protein – bound selenium and approximately the same dose of inorganic selenium on the selenium status. The higher efficacy of organic selenium was also confirmed by results of analyses of selected tissues. The concentrations of plasma Se at slaughter were lower, as a maximum was reached between days 28 and 35. It can therefore be concluded that, in terms of long – range effects, such treatment failed to improve the selenium status, but was successful in the short term.

Potential for commercialism

There is no doubt that Se enriched milk proteins and ingredients can be positioned in a range of markets for the maintenance and improvement of human health. It has been demonstrated that it is possible to produce milk products and casein products, which contain 3.0 to 4.9 and 2.4 to 6.6 ppm Se, respectively (DPI Kyabram). At these concentrations, they can be considered to be nutraceuticals suitable for prophylactic treatment of humans considered to be at risk of developing diseases, such as cancer of the colon (Rayman, 2000). The intent of such feeding strategies would be to achieve intakes of 1.0 to 2.0 ppm Se daily.

The following questions need to be answered to provide evidence to expand markets:

- What are the milk fractions that promote bioavailability or have high levels of bioactivity, and therefore, need to be maintained in a final product to ensure delivery of benefits?

- What is it about the chemical or physical nature of these compounds that delivers high bioavailability and, therefore needs to be protected or promoted during production or processing in order to deliver benefits.

Future work

Further work with human subjects is dependant on the findings in the present study. The proposed plan for clinical studies in sequence is as follows:

1. Short term work for fine tuning the most suitable dairy product for delivery, and the most suitable Se supplement (January-June 2006). Further development of relevant biomarkers for bowel health and colon cancer prevention will be proposed.

2. There is a need for safety studies, which would involve short term work in approximately 100 typical subjects and will compare selenised dairy protein with current Food and Drug administration (FDA) recommended alternative supplements (selenomethionine, selenised yeast) (January-June 2007).

3. A 1-3 yr study in several hundred people to study the impact of the product on colorectal adenomas (July 2007-December 2008). This is a large undertaking, but is the most persuasive study to gain acceptance.

Conclusion

Looking at GPx activity in plasma and liver samples, and Se concentration in plasma samples, there are higher levels of GPx activity and Selenium levels in pigs fed the HSeM diet (diet 1).

This suggests that the milk from the cows fed Sel-plex® is highly bioavailable, and may offer a means of delivering supplemented Se to humans.

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