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Anti-ageing Effects of Cysteine-containing Peptides Derived from Milk Whey Protein

Steffi Dudek and David C. Clark
DMV International

ABSTRACT

The trend towards ageing populations has been observed over many years in Europe and the US but has accelerated significantly in developed countries in Asia including Japan and South Korea. In the latter country the elderly population (65+) has increased 5-fold between 1960 and 2000 and this group will comprise 40% of the population by 2050. This creates a new socio-economic group with specific demands and considerable spending power. As ageing occurs a range of changes occur in the body that can be moderated by adjustments in nutrition. A significant body of evidence points to changes in the balance of glutathione synthesis and utilisation as people age. Glutathione is the most important natural anti-oxidant of the body and the amounts present can become limited by available cysteine in the diet. A cysteine-enriched peptide product, Cysteine Peption™ has been developed by DMV International for dietary supplement and food applications. A qualitative consumer trial has indicated benefits including improved sleep and more energy. Animal and clinical trials will be described that provide indications on bioavailability and possible mechanisms of action of Cysteine Peption™ with particular focus on the ageing population.

I. Introduction

The ageing of populations is a common trend in Europe, US and developed Asian countries. A combination of factors including economic development, industrialisation, decreasing birth rates and increased life expectancy due to improved nutrition and medical care have all contributed to this trend. Between 1960 and 2000 the population of South Korea has approximately doubled to 47 million. In this period, the elderly population (65+ years) has increased almost five-fold to 3.4 million [1]. Consequently, the proportion of elderly people in the total population has increased from 2.9% to 7.2%. The growth in the elderly community in South Korea is still accelerating. The proportion of population aged 60+ increased from 10.4% in 1998 to 12.3% in 2003 (Euromonitor). For comparison, in France it took 114 years for the percentage elderly to grow from 7% to 14% of the total population. On the other hand in Japan it took only 24 years. Recent estimates suggest that in South Korea it will take a shocking short 19 years, which would make it the fastest ageing country in the world. Further projections show that by 2050 40% of the population of South Korea will be over 65 years old (CSR Asia Weekly) and would surpass Japan as the most aged society in the world. These changes in population demographics are also accompanied by social changes. More and more elderly South Koreans are not living with their children but are choosing to live independently in retirement communities and have created a so called 'silver industry' of affluent silver-haired consumers with considerable disposable income. A study published by the Samsung Economic Research Institute forecasted that the silver industry was worth 27 trillion won in 2005 compared to 17 trillion won in 2000.

Corresponding author : David C. Clark, DMV International, P.O.Box 13, 5460 BA Veghel, The Netherlands, E-mail : steffi.dudek@dmv-international.com; david.clark@dmv-international.com; Tel: +31 413 372222

Ageing is characterized by a generalized impairment of physiological functions, which results in a decrease in ability to respond to a wide range of stress, an increased risk of development of age-associated diseases and an increased likelihood of death [2]. The risk of development of cardiovascular and heart diseases as well as cancer increases with age. Cellular senescence (impairment of the ability to proliferate) accompanies the ageing process, and very old individuals may develop muscular atrophy and muscle wasting.

A shift in the thiol / disulphide redox status (REDST) of the intracellular glutathione pool and the plasma cyst(e)ine and albumin pools, accompanied with a decrease in plasma thiol concentration has been found to be associated with ageing [3]. The reasons for this shift are very complex, but there is increasing evidence that a downward spiral exists which encompasses lower insulin responsiveness, leading to reduction in muscular protein synthesis, which in turn lowers the muscular cysteine clearance. Free cysteine is oxidized in the blood - resulting in the formation of reactive oxygen species (ROS). This depletion of plasma cysteine reduces the intracellular availability of sulphur containing amino acids to the tissues as its uptake by most cells is in the cysteine form. The oxidative shift in the thiol REDST leads to a dysregulation of redox-mediated signaling, which amongst other consequences affects insulin sensitivity, but also the regulation of the serum albumin synthesis and the oxygen transport capacity of the blood [3]. This in turn compromises muscular protein synthesis leading to a lower clearance accompanied with a higher plasma cysteine oxidation - and so forth.

When considering supportive nutrition for healthy ageing, it is logical to either improve cysteine availability and/or to re-establish the thiol REDST associated with younger ages. These simple supports are not as trivial as they sound, because increasing the concentration of available free cysteine has to take into account that it must not lead to further depletion of the thiol-related-redox-status.

Indeed, evidence exists that the administration of N-acetylcysteine (NAC) or other cysteine precursors diminished the symptoms of age-associated diseases in animals, and also showed promise in human clinical trials with cancer or HIV patients [4]. NAC is normally used for the treatment of a number of respiratory diseases and as a remedy after acetaminophen overdosing. However, high doses of NAC were also shown to act as pro-oxidant in healthy subjects [5].

Free cysteine is toxic and auto-oxidizes rapidly in the plasma, producing potentially toxic reactive oxygen species (ROS), especially in the presence of iron [6]. To avoid the intracellular toxicity of free cysteine, almost all of the non-protein cysteine is stored as glutathione (GSH). GSH is the major sulphydryl-containing compound in cells.

Glutathione is a tripeptide (γ-glutamyl-cysteinyl-glycine) which is synthesized in all cells. GSH cannot be taken up directly by most cells and needs to be degraded before membrane trafficking of the constituent amino acids. The limiting amino acid cysteine originates either from GSH itself, from dietary proteins or from methionine, which may be transformed into cysteine in the trans-sulphuration pathway. The GSH concentration in the liver is 5-10 mM [7], whilst in the plasma the GSH content is about 2 μM [8]. The cellular cysteine concentration is much lower than the Km values for the rate-limiting enzyme for the GSH synthesis, γ-glutamyl-cysteine synthetase. Therefor, already a moderate increase in intracellular cysteine is assumed to promote increased GSH synthesis [7].

Theoretically, the reducing capacity of the redox couple GSH/GSSG increases with the total GSH concentration [9]. This fact underlines the impact of the oxidative shift in the GSH-related REDST associated with the depletion of the absolute GSH concentration in elder subjects on their oxidative stress resistance. In other words, a preventive approach should both improve the redox status and increase the intracellular concentration of the redox buffers like GSH.

The liver is by far the organ with the highest

synthetic capacity for GSH and manages most part of the intra-organ exchange of GSH [10]. About 34 % of the blood passes through the intestinal system and the liver. The blood derived from the small intestine has to pass through the liver before it delivers its nutrients to the other parts of the body. In contrast to most other cell types, the liver is also able to absorb cystine, also referred to as cysteine disulfide, the oxidized form of cysteine. This fact may explain the pre-eminent role of the liver as a target organ for nutritional interventions in order to increase the systemic cysteine availability. It also points to the fact that liver dysfunction, either by infection, alcohol or other toxic damage, or as a consequence of adiposity, may disturb the systemic availability of cysteine and GSH as well as their redox status.

Glutathione is not only the store for systemic cysteine, but fulfills the following roles:

- The body's most abundant antioxidant and radical scavenger.
- A ligand for electrophiles in the liver detoxification system.
  Conjugation with GSH lowers the toxicity of xenobiotics and facilitates their excretion, but results in a net loss of GSH from the system.
- The major thiol-disulfide redox buffer. In this role, GSH modulates biological processes like *signal transduction*, e.g. those involved in the insulin responsiveness [11]; affects the *cell cycle regulation*, which has e.g. an impact on wound healing and repair [12] and is involved in the regulation of the *immune activity*.

Broad evidence exists showing that GSH is depleted in pathological states like cancer, HIV, hepatitis or diabetes [13, 14]. Inflammatory states like in arthritis induce oxidative stress which induce an oxidative shift of the GSH related REDST. Increasing amounts of prescribed medications for elder people, e.g. pain relievers, exert an additional exogenous stress factor on the GSH pool due to excretion losses after biotransformation of the drugs.

Ⅱ. Development of Cysteine Peption™ for healthy ageing

In order to provide a safe cysteine source for conversion by the body into glutathione by the body, DMV International developed Cysteine Peption™. This is a milk derived protein hydrolysate contains the highest cysteine concentration among available food proteins (6.5% on protein). We hypothesize that Cysteine Peption™ would be an ideal nutritional supplement for "successful ageing"-products because:

- The high cysteine content allows a convenient cysteine supply without a high additional protein load.
- Cysteine Peption™ is transformed into glutathione in the liver, the physiological cysteine storage form. The benefits for elderly subjects are obvious: increased GSH synthesis will increase the systemic cysteine availability and at the same time improve GSH dependent functions, like detoxification.
- Cysteine Peption™ is safe. More than 90 % of the cysteine exists as cysteine disulfide, thus Cysteine Peption™ doesn't exert an inherent pro-oxidant potential or toxicity. Since the synthesis of glutathione is feed back regulated, an overdose of cysteine or over-synthesis of GSH is not possible.
- Easily digestible proteins have shown benefits to counteract age-associated protein loss in elder subjects [15]. Cysteine Peption™, a protein hydrolysate, perfectly matches the characteristics of an easily digestible protein source.
- Cysteine Peption™ is applicable in different consumer product types, from pills and clinical formula to functional food products. It is therefore a very adaptable ingredient to design a whole range of different consumer products that meet the special needs of the elderly.

1. Animal data

The objective of our animal trials was to establish a link between Cysteine Peption™ consumption and liver glutathione levels and to assess whether there was evidence that Cysteine Peption™ provided any form of protection to the liver against toxic and oxidative damage.

Rats were fed for 14 days with a standard diet containing 20% casein protein in which the protein was partially replaced by Cysteine Peption™ or N-acetyl cysteine (NAC). The latter is used as a antidote against acetaminophen poisoning. The concentration of -SH in the liver was assessed for animals receiving different sulphur content in their diet and the results are shown in Fig. 1.

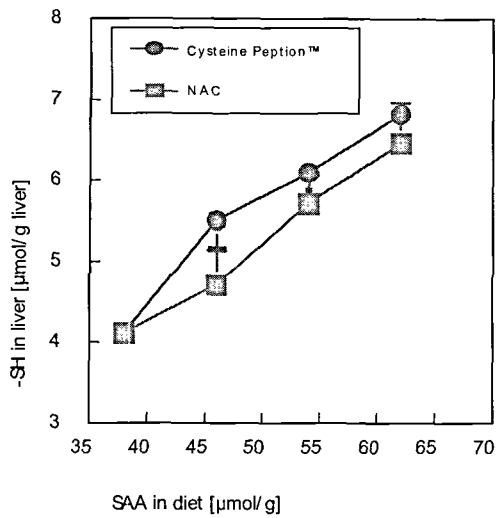


Fig. 1. Liver sulfhydryls [µmol/per gram liver tissue], depending on the concentration sulfur containing amino acids in the diet.

The results show that liver -SH was directly proportional to the Cysteine Peption™ or NAC content of the diet. In a second study, rats were fed a 20% casein or 14% casein plus 6% Cysteine Peption™ diet for 14 days. Six rats from each diet group were tested for liver GSH, immediately before an aminoacetophen (paracetamol) overdose (300 mg/kg body weight; approx. 100 mg/rat) was administered, at the beginning of a fasting period of 12 hours. Nine rats from each diet group were sacrificed and liver GSH was measured. Subsequently, the remaining 9 rats in each diet group were re-fed and subsequently sacrificed 12 hours later and the GSH was again measured. Histological assessments were made on the livers from the different cohorts with particular attention to the levels of vacuolated and necrotic cells and to the immune activity in the tissue as shown by the presence or absence of leucocyte clusters.

The experiment showed that increasing amounts of Cysteine Peption™ in the diet increases the glutathione concentration in rat livers up to a certain level but that the increased GSH could not prevent liver damage in rats if high concentrations of acetaminophen (APAP) were administered. However, in the recovery period, the GSH levels in the Cysteine Peption™ group were much higher than in the control group and exceeded the start level significantly. In line with this observation, the histology analysis indicated a better restoration of the tissue integrity in the Cysteine Peption™ group. (Fig. 2)

Our results support the hypothesis that Cysteine Peption™ has the potential to contribute to long term liver prevention and recovery after liver injury. Especially elder people should profit from Cysteine Peption™ because their bodies increasingly lose their ability to respond to toxic stress. Medications or alcohol ingestion, latent inflammations or heavy metal burden further stress the already compromised cysteine and glutathione concentration and their redox status.

The results of these studies provide indications that Cysteine Peption™ can support healthy liver function and contribute to the re-establishment of the cysteine and GSH homeostasis.

2. Consumer study

A small-scale qualitative consumer research study was conducted in the US with 13 consumers older than 50 years in order to assess benefits experienced with Cysteine Peption™. Suitable subjects were chosen via a questionnaire in which

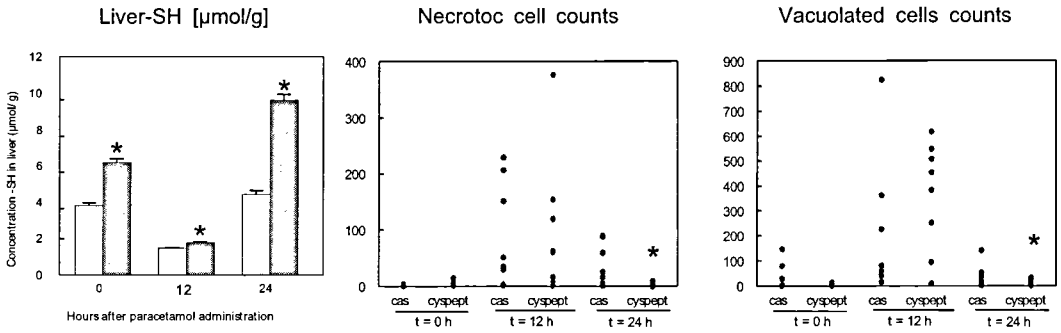
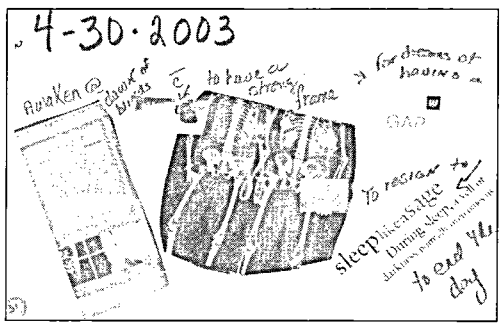


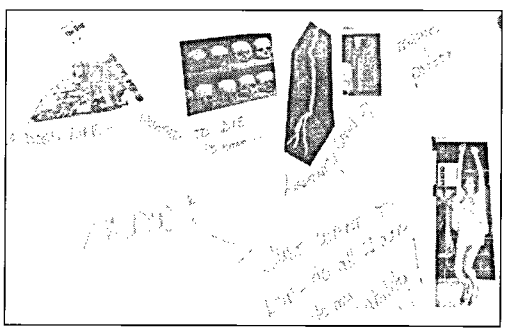
Fig. 2. Toxic challenge of rats with acetaminophen, animals fed with a casein diet or diet enriched with Cysteine Peptide. T = 0, 12 and 24 h after challenge.

age was one of the primary selection criteria. In this blind study, consumers took a daily dosage of Cysteine Peption™ of 3.3 g per day in tablet form for 4 weeks, equivalent to 200 mg of peptide bound cysteine per day. The effects were assessed by comparing their self-reported health and well being before and after taking Cysteine Peption™ through means of collages created with pictures that represented their feelings. An example of pre-use and post use results for a typical subject are shown in Fig. 3 (a and b).

In this blinded study, the subjects only were aware that the product was from a dairy company. In the pre-use, 'ideal health' collage (Fig. 3(a)) the subject apparently assumed the tablets of dairy ingredient would contribute to her having strong bones. She described wanting to wake up at the beginning of the day, her ambition to have a 'GAP BODY' suitable for wearing fashionable clothes and that she wanted to sleep well at the end of the day. In the follow up post use (Fig. 3(b)), she revealed how she really felt about herself and her health. She described about having a 'dog's life' and that sometimes she wanted to die. Lightening struck as a result of the magic potion she had taken (Cysteine Peption) and now she wanted to live and do all she can within her ability. This type of result was typical of those found. In summary, 9 out of 13 consumers reported that they felt more energetic, more motivated, and some reported improved sleeping patterns. The outcome of this consumer study was critical in our decision on design of the subsequent human clinical trial.



Pre-use of Cysteine Peption™



Post-use of Cysteine Peption™

Fig. 3. Consumer study: pre-and post use statemens of a 55 years old woman before and after ingestion of Cysteine Peption™ for 4 weeks.

3. Human study

Ethanol is a suitable model to study the effects of Cysteine Peption™ in healthy humans. Ethanol metabolism induces toxic and oxidative stress, which results in lipid peroxidation. Lipid peroxidation causes inflammation, and is one of the initial events in the generation of atherosclerotic plaques, which are involved in the development of CVD.

4. The metabolism of alcohol

Three routes of ethanol metabolism exist in the human body (Fig. 4).

- The oxidation of alcohol by alcohol dehydrogenase (ADH) in the liver, and, with less relevance, in the stomach. The oxidation product acetaldehyde is very toxic.
- The microsomal cytochrome P450 system (MEOS) especially at higher alcohol concentrations. This route is accompanied with the formation of free radicals and ROS, which contribute to an oxidative shift of the thiol REDST and a depletion of the GSH pool. Lipid peroxidation, DNA and protein damage are typical consequences. Alcohol furthermore induces CYP2E1, which may affect the toxicity of certain drugs like acetaminophen or other hepatotoxic agents [16, 17].
- the oxidation by catalase (minor importance)

In a very recent human clinical study, 20 healthy male volunteers received 40 g alcohol and 3.3 g Cysteine Peption™ or 40 g alcohol and 3.3 g placebo for three weeks, respectively. A mix of amino acids, corresponding to the composition of Cysteine Peption™ without cysteine, was used as the placebo. It was shown in previous experiments, that this amount of alcohol specifically induced an increase in urine F2-isoprostanes (F2IP), a product of lipid peroxidation. Lipid peroxidation may initiate tissue damage, which induces inflammation. C-reactive protein (CRP) is a marker for systemic inflammation and an independent predictor of cardiovascular events in healthy individuals. It was our hypothesis that Cysteine Peption™, by supporting liver GSH synthesis, would prevent lipid peroxidation and toxic damage following alcohol

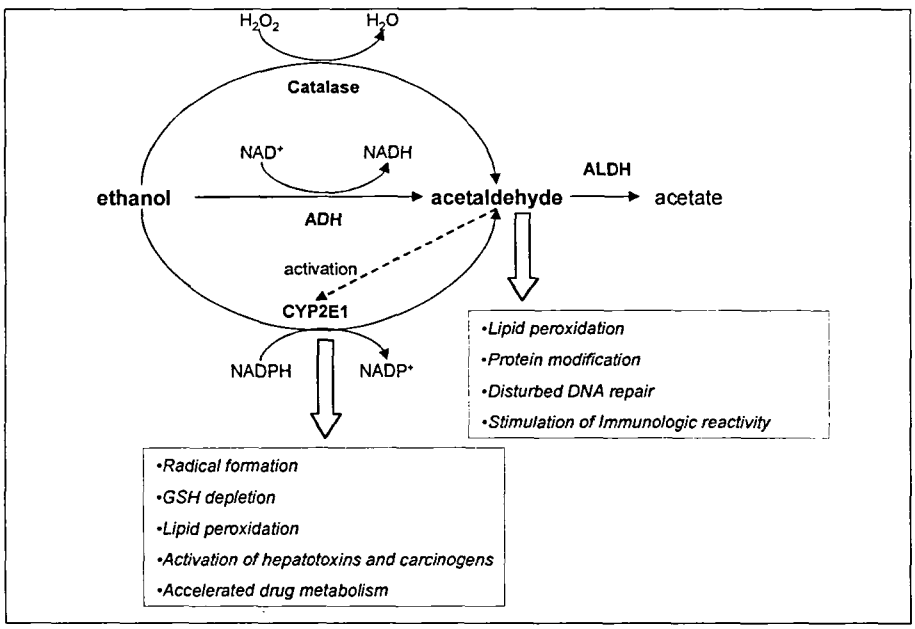


Fig. 4. The main routes of alcohol metabolism.

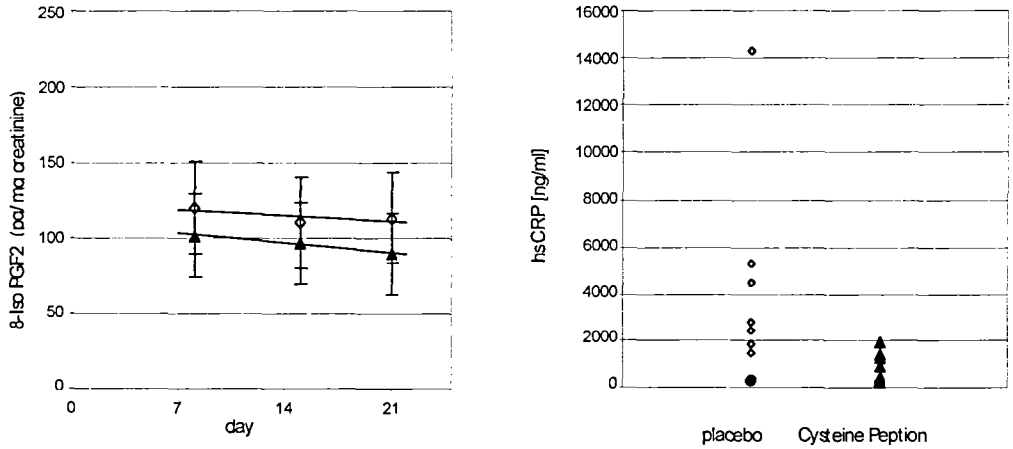


Fig. 5. Human study (20 healthy male subjects, mean BMI 26.9, mean age 43) F2-Isoprostane and hsCRP levels after 3 weeks alcohol ingestion and parallel administration of either placebo or Cysteine Peption™.

ingestion. Since the alcohol ingestion was far below a toxic level, we didn't expect a response in biomarkers of severe damage such as liver enzymes. Urine F2IP and plasma C-reactive protein (hsCRP), were the main parameters of interest.

The preliminary results showed, that the parallel ingestion of Cysteine Peption™ and alcohol led to lower hsCRP levels as compared with the placebo (Fig. 5). It was already known that low alcohol intake may lower the hsCRP values under certain conditions [19]. Apparently, Cysteine Peption™ amplified this effect. CRP responds very sensitively to nutritional interventions and glycaemic load. In our study, the subjects were under diet control, thus a treatment effect is very probable. In the given study configuration, the F2-isoprostanes did not respond to either treatment or placebo, suggesting that the F2IP marker was not sufficiently sensitive to detect an effect of Cysteine peption on lipid peroxidation in this study design. Since the analysis is not yet fully completed, no further interpretation may be given at the moment. More studies are required to understand the effect of Cysteine Peption™ on lipid peroxidation and inflammation in more detail.

Ⅲ. Conclusions

Influencing cysteine availability by nutritional intervention in ageing subjects is not trivial. More studies are needed to investigate the effects of Cysteine Peption™ on cysteine-or GSH dependent physiological functions with emphasis on ageing associated conditions and direct liver support. Nevertheless, the results of a consumer trial were very persuasive and the majority of participants consistently reported benefits. Animal studies have established a link between cysteine intake and liver glutathione levels. Whilst the elevated glutathione levels did not prevent oxidative damage to the liver in a severe challenge test, it did accelerate recovery of the tissue. Very preliminary data suggests a protective role of Cysteine Peption™ in the inflammatory pathways. Further clinical trials are necessary to establish a solid link between detoxification pathways and benefits such as improved sleep and energy levels reported by elderly consumers.

Ⅳ. Acknowledgements

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