

Ascorbate in the guinea pig lens: dependence on drinking water supplementation

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ABSTRACT.

Purpose: To investigate whether lens ascorbate concentration can be elevated with drinking water supplementation.

Methods: Pigmented guinea pigs received drinking water supplemented with L-ascorbate, concentration 0.00, 2.84, 5.68 or 8.52 mM for a duration of 4 weeks. In addition, the chow fed to all animals contained 125 mmol L-ascorbate per kg of chow. At the end of the supplementation period, the guinea pigs were killed. Each lens was extracted. The lens was processed and ascorbate concentration was measured using high performance liquid chromatography (HPLC) with 254 nm ultraviolet radiation detection. The data were analysed with regression.

Results: At the end of the test period, all lenses were devoid of cataract as observed by slit-lamp examination. All lenses contained a detectable concentration of ascorbate. Estimated 95% confidence intervals for mean animal-averaged lens ascorbate concentrations ($\mu\text{mol/g}$ wet weight of whole lens) per group were 0.51 ± 0.04 (0.00 mM; $n = 6$), 0.70 ± 0.18 (2.84 mM; $n = 6$), 0.71 ± 0.11 (5.68 mM; $n = 5$), and 0.71 ± 0.06 (8.52 mM; $n = 6$). Animal-averaged lens ascorbate concentration $[Asc_{lens}]$ ($\mu\text{mol/g}$ wet weight lens) increased with ascorbate supplementation in drinking water $[Asc_{water}]$ (M), in agreement with the model: $[Asc_{lens}] = A - Be^{-kAsc_{water}}$

Conclusion: Lens ascorbate concentration increases with drinking water supplementation in the guinea pig without cataract development. The currently presented method for measurement of whole lens ascorbate content is suitable.

Key words: ascorbate – guinea pig – lens – HPLC

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Introduction

The purpose of this paper was to investigate whether lens ascorbate concentration can be elevated with drinking water supplementation.

Ascorbic acid, or vitamin C, has two ionizable –OH groups, with $pK_{a1} = 4.25$ and $pK_{a2} = 11.8$. Ascorbate is the favoured form at physiological pH

(Halliwell & Gutteridge 1999). Therefore, we use the term 'ascorbate' throughout.

Ascorbate is an essential nutrient in both the human and the guinea pig, with the lens ascorbate concentration being much higher in diurnal than in nocturnal animals (Long 1961).

Ascorbate is of particular interest because of its role as an antioxidant in a number of tissues including the lens (Halliwell & Gutteridge 1999). It can prevent oxidative damage to protein, lipid and DNA in a number of tissues, including the lens (Sies et al. 1992; Reddy et al. 1998).

In vitro (Reddy & Bhat 1999; Sasaki 2000) and *in vivo* (Hegde & Varma 2004) studies in the guinea pig and rat show that ascorbate is protective against cataract formation and damage to lens constituents from various oxidative causes, including ultraviolet radiation (UVR).

To date, very few *in vivo* studies have described the role of ascorbate in preventing cataract in the guinea pig. *In vivo* studies have examined development of cataract (Malik et al. 1995) and lens DNA damage (Reddy et al. 1998) in scorbutic guinea pigs. However, both studies are indirect. The guinea pig is an appropriate UVR cataract model, as the ascorbate level in the aqueous humour and lens may be easily modulated by either dietary restriction or supplementation.

Evidence suggests that oral intake of ascorbate protects against cataract formation in the human lens (Leske

et al. 1991; Jacques et al. 1997). Further, lenses with increasing degree of cataract and browning secondary to protein oxidation were associated with lower ascorbate content (Tessier et al. 1998).

Besides functioning as an antioxidant in the lens, ascorbate in the aqueous humour attenuates UVR (Ringvold 1995).

Pharmacodynamics of ascorbate

In diurnal animals, including the guinea pig, the concentration of ascorbate in the aqueous humour is higher than in plasma. It is even higher in the ocular lens than in the aqueous humour because of a concentration gradient maintained by active transport (Garland 1991). Recently, the sodium-dependent ascorbate transporter (SVCT2) has been identified in the human lens epithelium (Kannan et al. 2001). The transporter may also exist in the guinea pig lens, although it has not yet been found.

Ascorbate is rapidly metabolized in the guinea pig, starting with oxidation to semidehydroascorbate (SDA) and further to dehydroascorbate (DHA) (Halliwell & Gutteridge 1999).

Measurement of ascorbate

Within the last 15 years, a method of measuring intraocular ascorbate utilizing high performance liquid chromatography (HPLC) with either electrochemical (Berger et al. 1989; Schell & Bode 1993; Watson et al. 1993; Richer & Rose 1998) or UVR (Hallström et al. 1989; Takano et al. 1997; Ringvold et al. 2000; Mody et al. 2004) detection has been described.

Detection of UVR is possible because of expressed absorption of UVR by ascorbate. High performance liquid chromatography with electrochemical detection has been applied to measure ascorbate in lens tissue from Emory mice (Taylor et al. 1995), guinea pigs and humans (Taylor et al. 1997). We decided to apply the technique of HPLC with UVR detection to ascorbate measurement in guinea pig lens samples processed and purified using ultrafiltration.

The current paper intended to determine a possible relationship between drinking water supplementation, in addition to food intake, and lens content of ascorbate in the guinea pig.

Material and Methods

Animals

Pigmented guinea pigs aged 5–11 weeks (HB Lidköpings Kaninfarm, Lidköping Sweden), weighing 304–638 g were used in the experiment. Ethical approval was obtained from the Northern Stockholm Animal Experiments Ethics Committee. The animals were kept and treated according to the *ARVO Statement for the Use of Animals in Ophthalmic and Vision Research*.

Experimental procedure

Groups of guinea pigs received drinking water supplemented with group specific L-ascorbate (Sigma-Aldrich, Stockholm, Sweden) concentration, at 0.00, 2.84, 5.68 or 8.52 mM, for a duration of 4 weeks. The drinking water was kept in bottles covered in dark plastic bags in order to prevent spontaneous oxidation of the ascorbate and was changed twice daily. Fresh ascorbate powder was added to each bottle at each water change. The amount of daily water uptake by each animal was monitored. In addition, the chow fed to all animals contained 125 mmol L-ascorbate per kg of chow (K1 Special, Lactamin, Stockholm, Sweden) for ethical reasons. Ascorbate was included in the diet to all guinea pigs in order to maintain the health of the animals.

Lens measurements

At the end of the supplementation period, the guinea pigs were killed with a pentobarbital overdose and lens ascorbate concentration was measured. The animals were not fasted before killing. Each lens was extracted in Ringer's acetate, photographed and wet weight measured.

Each lens was then homogenized in 1.0 ml 0.25% metaphosphoric acid with a tissue grinder (Hallström et al. 1989). Metaphosphoric acid prevents oxidation of ascorbate to dehydroascorbate because of high acidity and metal ion chelation (Washko et al. 1992; Koshiishi et al. 1998). The homogenate was centrifuged at 12 000 g for 10 min at 4°. Subsequently, the supernatant (800 µl) was ultrafiltered through a 5-kDa molecular weight cut-off membrane (Ultrafree CL; Millipore AB, Sundbyberg, Sweden) using centrifuga-

tion at 3830 g for 1 hour at 4° to provide a sample.

Ascorbate in the sample was separated on an ion-exchange, reversed phase column, 220 mm × 4.6 mm, 10 µm (Polypore H; Applied Biosystems, Foster City, California, USA), using 2 mM sulfuric acid pH 2.4 as the mobile phase. The sample (20 µl) was injected, without any pretreatment, by a refrigerated autoinjector set at 6° (CMA/200 Microsampler; CMA/Microdialysis AB, Stockholm, Sweden), using a 15.4-µl sample loop. The mobile phase was delivered at 0.3 ml/min using an isocratic HPLC pump (CMA 250; CMA/Microdialysis AB, Stockholm, Sweden). The exploring beam of the detection module consisted of radiation from a deuterium lamp that was spectrally limited with a monochromator to 254 nm, which is the classical wavelength for ascorbate measurement (Hallström et al. 1989). The absorbance of the column exit was measured using a UVR detector (L7400; Lachrom Merck Hitachi, Darmstadt, Germany). Before each series of HPLC measurements, a calibration procedure was run. The calibration procedure consisted of running a 10 equi-distance level calibration curve for the measurement range intended, to assure linearity. Then, the absorbance was obtained for two samples of 10 µM L-ascorbate (Merck, Darmstadt, Germany) diluted in 2.5% metaphosphoric acid and prepared from a stock solution. Finally, the absorbance of a 2.5% metaphosphoric acid-only blank was determined. If the blank resulted in measurable absorbance, the column was washed and the calibration procedure was repeated. The average absorbance for the two samples of 10 µM L-ascorbate was used to set sensitivity and, with this, ascorbate was calculated for each measured unknown sample considering a first order relationship between concentration of ascorbate and absorbance, omitting the zero order term.

Experimental design

Four groups of six guinea pigs each received drinking water supplemented with ascorbate to one of the concentrations 0.00, 2.84, 5.68 or 8.52 mM. One ascorbate measurement was obtained for each lens sample. Ascorbate content was measured in both lenses. Ascorbate

concentration was averaged for each animal.

Statistical parameters

Significance levels were set to 0.05 and confidence coefficients to 0.95.

Results

The guinea pig weights varied from 304 g to 638 g at the beginning of the study. All guinea pigs gained weight during the study (Fig. 1).

The 95% confidence interval for the mean weight increase from day 1 to day 30 was $30.9 \pm 3.8\%$. The difference in weight gain among the four ascorbate supplementation groups was not statistically significant according to an analysis of variance.

All lenses were devoid of cataract under slit-lamp examination both at the beginning and the end of the experiment. Figure 2 shows lenses for each of the four groups of drinking water supplementation.

Figure 3 shows a typical chromatogram for ultrafiltered whole lens homogenate.

Retention time for ascorbate was 6.1 min under the HPLC conditions used in the experiment. Ascorbate was separated from other absorbing substances. The peak shape and symmetry allow for identification of the ascorbate peak.

All lenses contained a detectable concentration of ascorbate (Fig. 4). The 95% CIs for the mean animal-averaged lens ascorbate concentrations (μmol ascorbate/g wet weight of whole lens) per group were 0.51 ± 0.04 (0 mM; $n=6$), 0.70 ± 0.18 (2.84 mM; $n=6$), 0.71 ± 0.11 (5.68 mM; $n=5$), and 0.71 ± 0.06 (8.52 mM; $n=6$). The figures for lens ascorbate are means \pm CI.

One animal in the 5.68 mM group was excluded from data analysis because the reading of ascorbate concentration in one of the lenses was more than twice that of all other measurements in the group. The fact that the 95% CI for mean lens ascorbate at 0 mM addition of ascorbate to the drinking water excludes zero implicates a statistically significant amount of ascorbate in the lens, with only the food baseline intake of 125 mmol L-ascorbate/kg chow.

The animals were 5–11 weeks of age at the beginning of the experiment. There were no age-related variations in lens ascorbate concentration in any of the four drinking water supplementation groups (determined by means and 95% CIs; data not shown).

The drinking water was changed twice daily and fresh ascorbate added, and the amount of water consumed by each animal was measured. Differences in the amount of water consumed by each animal do not explain the variation in lens ascorbate concentration (data not shown), especially for the 2.84 mM group.

Inspection of the data suggests that animal-averaged lens ascorbate concentration $[ASC_{lens}]$ increased with ascorbate supplementation in drinking water $[ASC_{water}]$. The increase in lens ascorbate concentration reached a saturation level at higher drinking water concentrations. Lens ascorbate concentration increased from a baseline level, and at some point the lens became saturated. Therefore, the data were fit assuming the model:

$$[ASC_{lens}] = A - Be^{-kASC_{water}}$$

Here, A is the saturation level of ascorbate concentration in the lens with drinking water supplementation, B is the increase of lens ascorbate from baseline to saturation, and k is a meas-

ure of the increase in lens ascorbate concentration as a function of ascorbate concentration in supplemented drinking water.

Non-linear regression according to the assumed model (Equation 1) provided a good fit ($r^2=0.98$). The estimates of the parameters and their estimated standard deviations were as follows. The saturation level, A, was estimated to $0.71 \pm 0.03 \mu\text{mol}$ ascorbate/g wet weight of lens and the increase of lens ascorbate from baseline to saturation, B, was $0.20 \pm 0.05 \mu\text{mol}$ ascorbate/g wet weight of lens. The baseline lenticular content of ascorbate, A–B, was calculated to $0.51 \mu\text{mol/g}$ wet weight of lens. The saturation rate, k, was $0.98 \pm 1.49 \text{ M}^{-1}$ ($1/k = 1.02 \text{ M}$).

Discussion

The current study aimed at investigating whether lens ascorbate concentration in the guinea pig can be increased by drinking water supplementation.

As our goal was to elevate lens ascorbate levels, we chose to supplement the drinking water with large amounts of ascorbate in order to increase lens ascorbate concentration to a maximum. We used high amounts of ascorbate supplementation, up to 10-fold the amount used by Taylor et al. (1997) in a study on vitamin C in guinea pig eye tissues in relation to intake. The reason for not choosing to feed guinea pigs a low ascorbate or ascorbate-deficient diet was that ascorbate deprivation for 2–3 weeks in two studies caused guinea pigs to become ill and lose weight (Malik et al. 1995; Reddy et al. 1998). The idea was to see whether we could achieve modulation of lens ascorbate levels through drinking water supplementation and whether the modulation could be used to study cataract models, including UVR.

We chose to feed the guinea pigs drinking water supplemented with ascorbate in addition to standard chow for a period of 4 weeks. The purpose of feeding for 4 weeks was to achieve a steady state lens ascorbate concentration.

Our selection of the method for ascorbate measurement from among other methods published (Omaye et al. 1979) was based on the premise that HPLC allows accurate separation, and that UVR provides specific detection

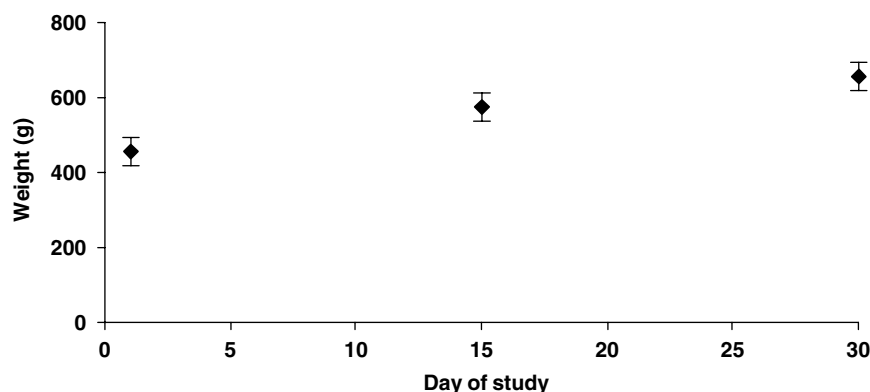


Fig. 1. Guinea pig weights on days 1, 15 and 30 of the study. 95% CIs for the mean are shown.

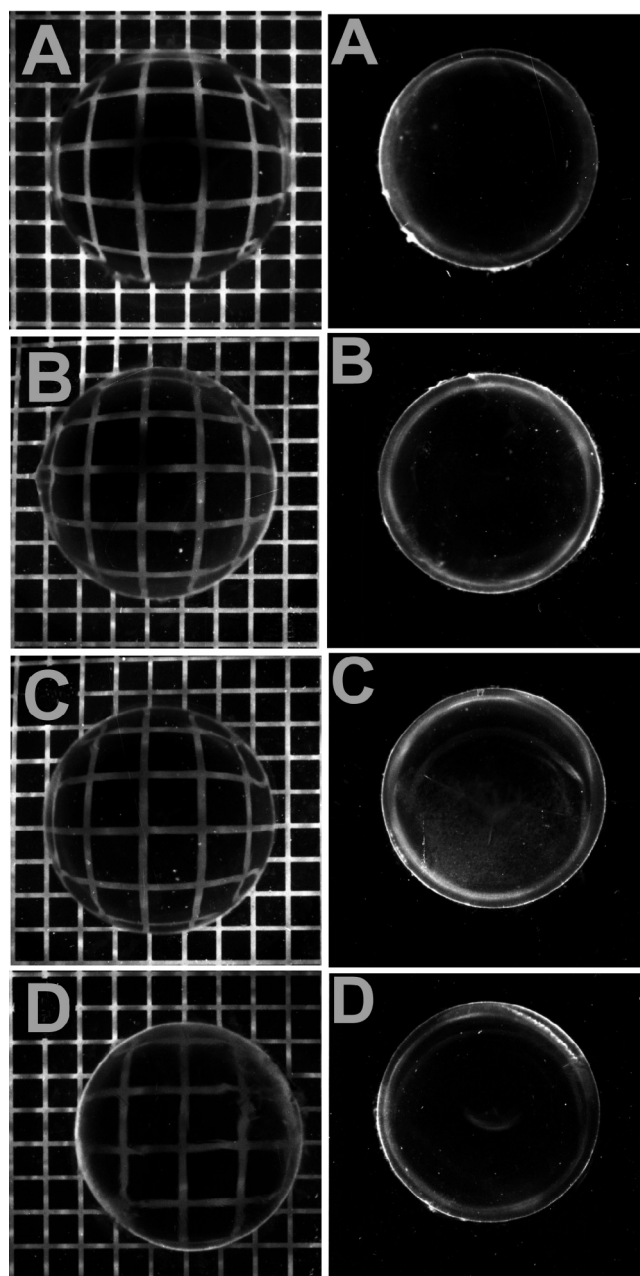


Fig. 2. Lens photographs using both bright field and dark field illumination for guinea pigs receiving drinking water supplemented with varying amounts of ascorbate in addition to standard chow. (A) 0 mM; (B) 2.84 mM; (C) 5.68 mM, and (D) 8.52 mM. Grid square diameter is 0.79 mm.

with high sensitivity for ascorbate in biological tissues (Johnsen et al. 1985; Hallström et al. 1989).

The adapted method for sample preparation of ascorbate was found to be adequate. The current HPLC method has the advantage over previously described methods of allowing for the detection of small concentrations of ascorbate in biological tissues, less than $0.06 \mu\text{M}$ in the injected sample (Hallström et al. 1989).

When processing the lens sample for ascorbate measurement, it is important

to add an agent to the sample to prevent metal catalysed oxidation of ascorbate. The lens samples in the study were prepared in metaphosphoric acid. Metaphosphoric acid prevents oxidation of ascorbate to dehydroascorbate because of high acidity and metal ion chelation (Washko et al. 1992; Koshiishi et al. 1998).

The current HPLC method is highly precise in the measurement range for lenses, based on experiments on rat lenses using both external and internal controls. The external controls, in

which ascorbate solutions ranging from $0 \mu\text{M}$ to $20 \mu\text{M}$ were prepared and measured on HPLC, followed a linear increase with a significant regression ($r^2 > 0.99$; data not shown). We have already shown that external calibration provides results equivalent to those that emerge when an internal standard addition technique is used (Mody et al. 2004). Therefore, external calibration was used here.

The quantitative recovery of ascorbate in the measurements was determined earlier on three rat lenses by re-extracting the pellet after centrifugation of the lens homogenate (Mody et al. 2004). The re-extracted pellet was then re-centrifuged. The ascorbate concentration in the supernatant after re-extracting the pellet was 46% of that in the supernatant of the lens homogenate. This finding indicates that ascorbate in the pellet is released into solution upon re-extraction.

We have also shown that our HPLC separation and UVR detection reassuringly separates reduced ascorbate from its oxidation product dehydroascorbate (Mody et al. 2004).

No cataract developed in any of the supplemented animals in the experiment. This finding is important considering the potentially dual role of ascorbate. Ascorbate normally functions as an antioxidant in the lens and does not glycate proteins by itself. However, at high concentrations in the presence of metals, when significant oxidation to dehydroascorbate occurs, ascorbate may serve as a pro-oxidant through protein ascorbylation, resulting in the formation of advanced glycation end-products (Cheng et al. 2002). Because no cataract developed in any of the supplemented animals, the results from the study are more consistent with the antioxidative role of ascorbate than its pro-oxidant capacity.

We found that there is a baseline lenticular content of ascorbate (Fig. 4) in guinea pigs receiving ascorbate only in their chow. The baseline level, $0.51 \pm 0.06 \mu\text{mol/g}$ wet weight of lens, is in the same order as those reported in the literature for guinea pigs receiving a standard diet, ($0.65 \mu\text{mol/g}$ wet weight of lens) (Varma 1991) and ($0.67 \mu\text{mol/g}$ wet weight of lens) (Reddy et al. 1998).

In the guinea pig, uptake of ascorbate by the lens from the aqueous humour, and by the aqueous humour from the plasma, occurs by active

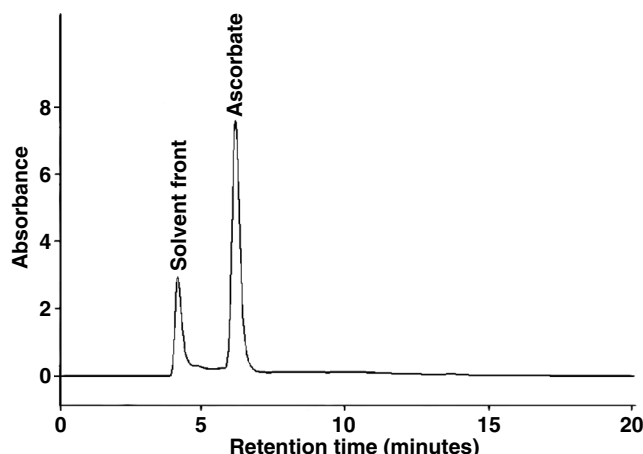


Fig. 3. Chromatogram for ultrafiltered whole lens homogenate. Ultraviolet detection at $\lambda = 254$ nm.

transport (Garland 1991). The currently found saturation of lenticular ascorbate secondary to drinking water supplementation (Fig. 4) agrees with previous findings in the guinea pig (Berger et al. 1988). However, we used a higher amount of ascorbate supplementation. These two findings indicate that the active transport is saturable.

The increase in lenticular ascorbate concentration with dietary supplementation in the guinea pig was significant: approximately 40% from group 1 to group 4. The increase is important as it could be used to study the preventive effect of lenticular ascorbate against oxidatively- and photochemically-induced cataract *in vivo*. We plan to study the modulatory effect of ascorbate on UVR-induced cataract in the guinea pig *in vivo*.

In conclusion, grinding of the lens in metaphosphoric acid, ultrafiltration of the lens homogenate and subsequent HPLC with UVR detection can be used to measure ascorbate in the guinea pig lens. The guinea pig lens has a detectable quantity of ascorbate with standard dietary ascorbate intake. Lens concentration of ascorbate increases to saturation with increased drinking water supplementation without cataract development.

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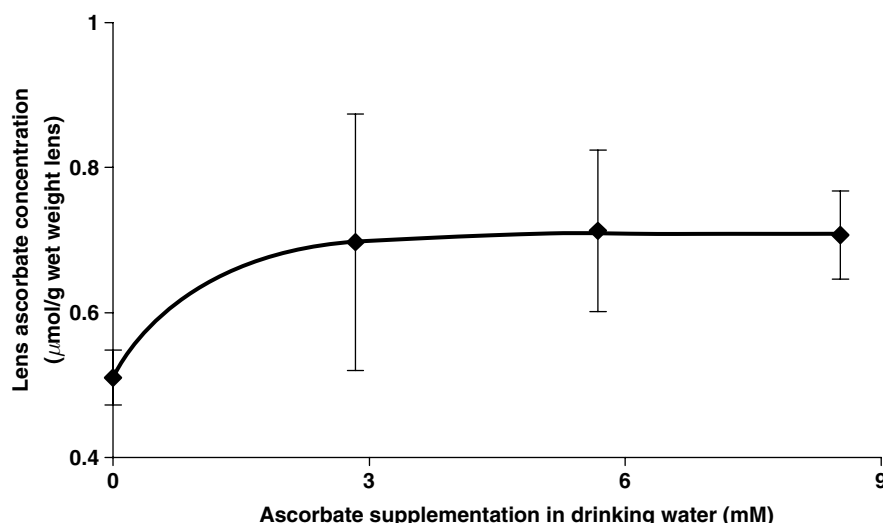


Fig. 4. Lens ascorbate concentration plotted against drinking water supplementation. For each group, 95% CIs for means are shown. The line shows the best fit to a model expressing exponentially declining increase.

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