In Vitro and In Vivo Reduction of Erythrocyte Sorbitol by Ascorbic Acid

Vinson, J.A., Staretz, M.E., Bose, P., Kassm, H.M. and Basalyga, B.S.

Abstract

The *in vitro* accumulation of sorbitol by Human erythrocytes incubated in a physiological glucose medium was found to be strongly reduced by the addition of ascorbic acid (AA). A maximal inhibition of sorbitol in the erythrocytes of 98.3% occurred when the concentration of AA was at its peak in the cells. After incubation, the erythrocyte sorbitol was found to be inversely correlated with the concentration of AA in the erythrocytes. A human supplementation study was conducted with 10 normoglycemic subjects. Each was given 500 mg/day AA alone or in a citrus fruit medium. Each supplementation lasted 2 weeks and was followed by a 10 day washout. The citrus fruit medium produced a significantly greater increase in erythrocyte AA compared with AA alone. AA alone and citrus fruit medium decreased erythrocyte sorbitol 12.6 and 27.2%, respectively, with the latter being significantly more effective. In a study with 4 subjects, 2000 mg/day AA resulted in a reduction in erythrocyte sorbitol of 56.1%. As in the *in vitro* study, there was an inverse relationship between erythrocyte AA and sorbitol. 2000 milligrams of AA per day (AA or citrus fruit medium) was given to 8 diabetic subject in a preliminary 3 week supplementation trial in which erythrocyte sorbitol levels were decreased by 44.5% These results suggest that AA supplementation for diabetic subjects may provide a simple means of preventing and ameliorating the complications of diabetes without the use of drugs.

Introduction

Sorbitol, produced by the reduction of glucose in a reaction catalysed by the enzyme aldose reductase, has been implicated as a causative factor in the long term complications of diabetes. These complications include; cataracts, neuropathy, retinopathy and nephropathy. They arise in tissues in which the transport of glucose is insulin dependent. Sorbitol accumulates in proportion to the extracellular concentration of glucose in sensitive tissues: notably the lens, retina, aorta, renal pailla and peripheral nerve [1]. Drugs that lower sorbitol concentrations by inhibiting aldose reductase allow new treatment possibilities for the complications of diabetes [2,3].

Ascorbic acid (AA) concentration and turnover in the plasma of diabetic individuals have been found to be lower than normal [4,5]. Glucose has been found to inhibit the *in vitro* uptake of AA and its reduced form, dehydroascorbic acid in granulocytes [6], endothelial cells [7], neutrophils and fibroplasts [8,9], and erythrocytes [10]. Chen et al. [11] have recently shown that in diabetic subjects intravenous glucose administration and the resultant hyperglycaemia caused a marked depletion of leukocyte AA that did not recover within 1 hour, which was in contrast with findings from normal subjects. The transport of AA has been shown to be enhanced by insulin [12,13]. Thus, diabetic individuals who have low insulin and high glucose levels may be at a double disadvantage with respect to AA uptake and storage and would be expected to develop a "local scurvy" as hypothesised by Mann and Newton [14].

Our group has recently found that supplemental AA slowed down the progression of galactose induced cataracts in rats, a diabetic cataract animal model [15]. AA also

was found to diminish the production of dulcitol, the galactose analogue of sorbitol, in lenses incubated in a high galactose medium. In a single dose human study, 500 mg of AA in a citrus fruit media was found to absorb better than AA alone [16]. These findings led us to investigate the effect of AA and citrus fruit media on *in vitro* and *in vivo* sorbitol production in human erythrocytes.

Research Design and Methods

Forms of AA: AA crystals were obtained from Fisher (Pittsburgh, PA.). AA tablets, 500 and 1000 mg, were distributed by Fay Drugs (Liverpool, NY.). Citrus fruit medium, a tan powder, was obtained, and contained 25.1% AA, 19.5 % bioflavonoids, 25% carbohydrates and 27% protein. Caplets of 250 mg AA or citrus fruit medium were obtained. The ascorbate in the citrus fruit medium was measured in our laboratory by high performance liquid chromatography (HPLC) after protein precipitation [17]. The bioflavonoids in the citrus fruit medium were identified and quantified by HPLC and found to contain 81.8% naringenin and 18.2% hesperidin [18].

Incubation: Whole blood was collected in EDTA tubes from a single adult volunteer by fingerstick sampling with a lancet. The blood was immediately centrifuged to separate the erythrocytes from the plasma. The erythrocytes were washed 3 times with isotonic saline and centrifuged.

A modification of the incubation procedure of Malone [19] was used. One hundred microlitres of erythrocyte were immediately added to 1 ml of Hanks' balanced salt solution was used for the AA samples. These solutions were incubated at 37°C for 3 hours in an atmosphere of 95% air / 5% CO_2. The cells were centrifuged, washed with saline and centrifuged again. The protein was precipitated with 2 ml of cold 6% perchloric acid. The solutions were stored at -20°C until analysis.

Human Supplementation: Ten subjects (3 women, 7 men) aged 18-44 years, volunteered for the study with informed consent. They were normoglycemic (4.03 ± 0.72 mM) determined by hexokinase assay of fasting plasma (θ (Sigma)). Each subject was tested in the morning after an overnight fast. Blood was collected with a fingerstick lancet and the separated erythrocytes were stored at -20°C after protein precipitation with 6% perchloric acid.

Each subject took 500 mg AA/day in the form of AA tablets or citrus fruit medium powder in a random crossover design. The citrus fruit medium was measured by volume. The subjects were instructed to take the supplement at breakfast. After 2 weeks of supplementation and an overnight fast, blood samples were obtained from the subjects 24 hours after the last supplementation when AA levels had returned to baseline [16]. A 10 day washout was followed by another sampling. The second supplement was then given for 2 weeks and another sample taken. A paired Student's *t*-test was used for statistical analysis.

A second supplementation study involved 4 normoglycemic men, aged 18-44 years, from the previous study. Samples were obtained as before, and then subjects were given 2000 mg AA/day in the form of 1000 mg AA tablets. They were instructed to take one tablet with the morning meal and one with the evening meal. After 2 weeks samples were obtained again.

Eight diabetic (5 insulin-dependent and 3 non insulin-dependent) subject participated in a 3 week supplementation study after giving informed consent. There were 6 women and 2 men with an average (±SD) age of 47 ± 17 years (range 16-68) and an

average duration of diabetes of 15.2 years. The average (±SD) fasting plasma glucose for the subjects was 9.17 ± 2.97 mM. The subjects were divided into two groups for supplementation. Each group contained 3 women and 1 man, with no significant difference in age or duration of diabetes. The subjects took four 250 mg caplets of AA in the form of AA or citrus fruit medium twice a day with meals for a total daily dose of 2000 mg of AA. Sampling was done before and 3 weeks after supplementation.

Analysis of AA: A modification of the fluorometric method of Hubman et al [20] was used for analysis of AA in erythrocytes. The assay uses 1,2 napthoquinone-4-sulfonic acid as the fluorometric reagent and measures only unoxidised AA in the erythrocytes. The supernatant from the protein precipitation of the erythrocyte was mixed with an equal volume of 1 M K_2CO_3 before analysis.

The concentration of AA in the erythrocyte of the single person used for the *in vitro* study was 29.6 ± 4.1 μM, which is similar to the average found in unsupplemented normal individuals (43 μM) reported by Evans et al. [21].

Analysis of Sorbitol: The enzymatic assay of Clements et al [22] was used to measure concentration of sorbitol in erythrocytes. Sorbitol is converted to fructose with sorbitol dehydrogenase and the resultant reduction of NAD+ to NADH is measured fluorometrically. A Perkin-Elmerm model LS-3 digital fluorometer was used with a NAD+ concentration of 0.2 mM. The sensitivity of the method was found to be 0.5 nmol/ml. Adding AA did not affect the development of the fluorescence. The concentration of sorbitol in the erythrocytes used for the incubation study was 17.6 nmol/ml, which is within the reported normal range of 10-27 nmol/ml [23].

Results
The data from the multiple incubations of the same erythrocyte with different concentrations of AA in the medium are shown in Tables 1. Hank's solution has a concentration of 5.56 mM glucose in the medium, which stimulates normoglycemia.

Medium AA and Erythrocyte AA: The concentration of AA in the erythrocyte should increase with increasing concentration of AA in the medium and then reach a plateau because the transport process is a saturable one [9]. However, the data from table 1 shows that the AA concentration in the erythrocyte is biphasic, reaching a maximum and declining. When the concentration of AA in the medium is 10-100 times higher than physiological concentration (284 and 2840 μM), the erythrocyte AA is constant and not significantly different from that found in erythrocyte incubated without AA in the medium.

Table 1: Effect of different concentrations of ascorbic acid (AA) in medium on concentration of AA and sorbitol.

Medium AA (μM)	n	Erythrocyte AA (μM)	Erythrocyte Sorbitol (nmol/ml)
0 (Control)	6	14.7 ± 0.4	46.4 ± 1.6
2.84	6	15.7 ± 0.4 *	10.3 ± 2.0 *
14.2	4	16.8 ± 0.3 *	6.7 ± 1.9 *
28.4	6	17.7 ± 0.9 *	0.8 ± 0.5 *
142	3	16.0 ± 0.4 *	11.2 ± 0.3 *
284	4	14.7 ± 0.4	31.5 ± 1.7 *
2840	3	14.4 ± 0.0	27.8 ± 2.4 *

Erythrocytes incubated at 37°C for 3 hours in Hank's solution containing 5.56 mM glucose. Values are means ± SD. n = number of replicate experiments.
* P < 0.001 by 2 tailed Student's *t*-test compared with control.

Figure 1: Effect of medium ascorbic acid (AA) concentration on reduction of erythrocyte (red blood cell) sorbitol relative to control with no AA in medium.
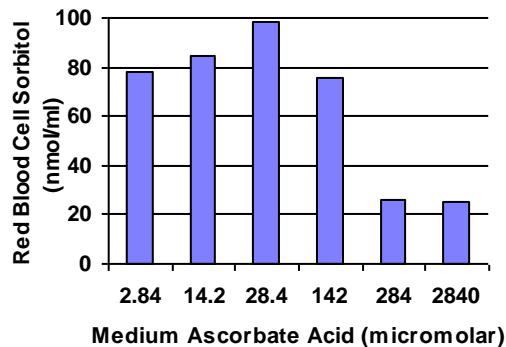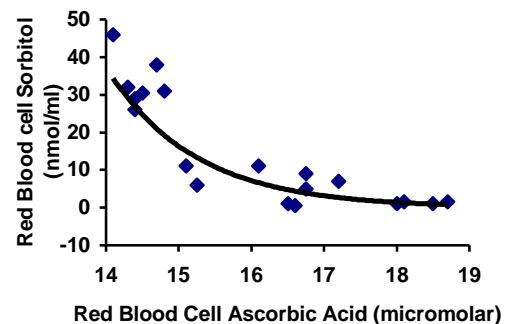
Figure 2: *In vitro* relationship between erythrocyte ascorbic acid and red blood cell sorbitol.

This may be attributed to the haemolytic effect of high AA concentrations on erythrocytes, which results in the destruction of AA [24]. As expected, a greatly increased haemoglobin concentration in the medium was observed after incubation when the initial AA concentration was much higher than the physiological concentration. Another mechanism for the lack of increase of erythrocyte AA at higher than physiological levels of AA in the medium is the increasing efflux of AA, as seen by Khatami et al. [25] and Socci and Delamere [26] at high AA concentration in the medium of cultured cells.

Sorbitol and AA: The concentration of sorbitol in the erythrocytes after incubation without AA (46.4 nmol/ml) is much greater than the preincubation value of 17.6 nmol/ml, an effect also observed by Morrison et al. [27]. All incubations with AA produce a significant decline in erythrocyte sorbitol relative to the control without added AA (Table 1; P < 0.001). Fig. 1 shows a biphasic effect of AA on the percentage inhibition of sorbitol relative to the control, which contained no AA in the medium. Almost complete inhibition of sorbitol (98.3%) occurs at a medium AA concentration of 28.4 μM. A similar level and pattern of inhibition was seen when citrus fruit medium was incubated in the medium instead of AA (data not shown).

The biphasic effect of AA on sorbitol disappears when the concentration of AA in the erythrocyte is plotted against the concentration of sorbitol in the erythrocyte (Fig. 2). An inverse exponential relationship exists between erythrocyte AA and erythrocyte sorbitol. At low values of erythrocyte AA, the concentration of sorbitol should increase exponentially, indicating by analogy that in scurvy or individuals with low erythrocyte AA the sorbitol levels will be high. At higher concentrations of erythrocyte AA, the sorbitol levels approach 0 as a limit. Note that the maximum erythrocyte AA concentration corresponds to the greatest sorbitol reduction (Table 1). This *in vitro* data indicates that AA supplementation should lower erythrocyte sorbitol.

Human supplementation studies with normal subjects: The average baseline value for the 10 subjects' erythrocyte AA was 32.8 μM with a range of 29.7 - 36.8. The average baseline concentration of erythrocyte sorbitol was 19.6 nmol/ml with a range of 17.4 - 22.1 and is similar to that reported by Akgun et al. [23]. For all

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supplementations, after washout, the AA and sorbitol concentrations were not significantly different from the baseline values.

The results of the first supplementation with 500 mg of AA, as in the form of AA or citrus fruit medium, are shown in Table 2. Individual percentage changes were calculated and the data averaged. Both forms of AA significantly increased erythrocyte AA ($P < 0.001$ for AA, $P < 0.01$ for citrus fruit medium). The average per cent increase in erythrocyte AA was 35.5% for AA and 57.5% for citrus fruit medium. In comparing these two forms, citrus fruit medium produced a significantly greater per cent increase in erythrocyte AA ($P < 0.05$). The greater effectiveness of citrus fruit medium in elevating AA can be attributed to its greater bioavailability as shown in the previous human absorption study [16].

Table 2: Effect of 500mg/day of ascorbic acid (AA) supplementation as AA alone or in citrus fruit medium for 2 weeks on erythrocyte AA and sorbitol of 10 normo glycemic subjects.

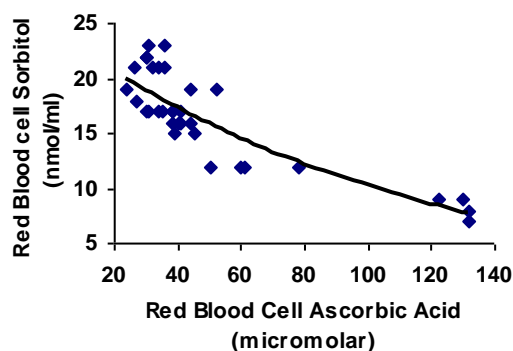| Erythrocyte AA (μM)			
Supplement	Before	After	Change
AA	33.2 ± 2.8	44.8 ± 5.4 *	+ 35.4 ± 19.4%
Citrus Fruit Medium	32.4 ± 3.8	48.8 ± 15.4 +	+ 57.5 ± 34.8% +
Erythrocyte Sorbitol (nmol/ml)			
Supplement	Before	After	Change
AA	19.5 ± 1.5	17.0 ± 1.4 *	- 12.6 ± 4.9%
Citrus Fruit Medium	19.7 ± 1.4	14.3 ± 2.5 *	- 27.2 ± 10.5% **

Values are means ± SD.
* $P < 0.001$, + $P < 0.01$, vs. before supplementation.
+ $P < 0.05$, ** $P < 0.01$, citrus fruit medium vs. AA.

The most dramatic difference between the two forms of AA is seen in the sorbitol data (Table 2). The AA produced a small but highly significant 12.6% reduction of erythrocyte sorbitol (range 8.1 - 19.9%). Even though the effect was small, all of the 10 subjects experienced a reduction. Citrus fruit medium caused a highly significant 27.2% decline in erythrocyte sorbitol (range 15.1 - 43.0%) with all subjects experiencing a decline. The citrus fruit medium produced over a twofold greater reduction than the AA, and the difference between the two forms was significant ($P < 0.01$). The greater ability of citrus fruit medium to lower erythrocyte sorbitol can be attributed to its greater effectiveness in increasing erythrocyte AA.

Figure 3: *In vivo* relationship between erythrocyte (red blood cell) ascorbic acid and red blood cell sorbitol.

The four subjects who took 2000 mg AA/day had a change in erythrocyte AA from 33.3 ± 2.68 to 130 ± 4.8 μM, an average increase of 291%. At the same time the

erythrocyte sorbitol went from 19.4 ± 1.5 to 8.1 ± 2.0 nmol/ml, an average decrease of 56.1%. This dosage of AA produced a significantly greater increase in erythrocyte AA and reduction of erythrocyte sorbitol than did either of the two forms of AA at 500 mg/day (P < 0.001). The erythrocyte AA and sorbitol data were pooled for all the supplementation studies with normal subjects (Fig. 3). The points define an exponential curve that has a shape very similar to that seen in Fig. 2 for the *in vitro* study except that erythrocyte sorbitol appears to approach 8 nmol/ml as a limit rather than 0.

***Diabetic Supplementation*:** The results of the supplementation of 8 diabetic subjects with 2000 mg AA/day in the form of AA or citrus fruit medium for a 3 week period are shown in Table 3. The data from the AA and citrus fruit medium groups were combined because there was no significant difference between the groups. Both forms of AA produced a significant increase in plasma AA, averaging 64.9% for the combined groups (P < 0.001), AA supplementation produced an average decrease in erythrocyte sorbitol of 44.5%, a change that was highly significant. As in the *in vitro* and *in vivo* normoglycemic studies, the diabetic study also showed an inverse relationship between AA and erythrocyte sorbitol.

Table 3: Effect of 2000 mg/day of ascorbic acid (AA) supplementation as AA alone or in citrus fruit medium for 3 weeks on plasma and erythrocyte sorbitol of 8 diabetic subjects.

Supplement	n	Plasma AA (μM)		Erythrocyte Sorbitol (nmol/ml)	
		Before	After	Before	After
AA	4	31.7 ± 15.0	54.4 ± 10.0 *	50.0 ± 17.7	26.1 ± 17.2 *
Citrus Fruit Medium	4	35.0 ± 8.3	55.6 ± 5.0 *	52.3 ± 24.8	30.7 ± 14.3
Combined Groups	8	33.3 ± 11.1	54.9 ± 7.2 +	51.2 ± 20.0	28.4 ± 14.9 +

Values are means ± SD. n = number of subjects in each group.
* P < 0.05, + P < 0.01, + P < 0.001, vs. before supplementation.

Discussion

The results of both the *in vitro* incubating of erythrocytes with normal concentrations of glucose and *in vivo* supplementation studies with normoglycemic subjects indicate that increasing the erythrocyte AA produces a greatly diminished sorbitol concentration. Although only erythrocytes were studied, it has been found by Malone et al. [28] that erythrocyte sorbitol correlates with sorbitol in the lens and sciatic nerve, two sites for diabetic complications. Thus, AA may prove useful for the prevention and treatment of the complications of diabetes. It has already been shown to be effective in retarding the progression and accelerating the regression of galactose cataracts in rats, an animal model of diabetic cataracts [15].

As a result of the greater effectiveness of 2000 vs. 500 mg AA/day given to normal subjects, a preliminary 3 week supplementation study was conducted with eight diabetic subjects who were given 2000 mg AA/day or citrus fruit medium. A comparison of this dosage of AA on the plasma AA of normal and diabetic subjects is shown in Fig. 4. In contrast to other reports, the diabetic subjects in this study had identical concentrations of plasma AA to that of the normal subjects (33.3 μM). This may simply be due to the small number of subjects in this study. The 65% increase in the plasma AA of diabetic subjects was significantly less (P < 0.001) than the 291% increase seen in normal subjects in this study and less than the 162% increase found in normal subjects by Evans et al. [21]. This difference may be attributed to the hyperglycaemic conditions in the diabetic subjects. Hyperglycaemia

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has been shown to inhibit the uptake of AA in tissues (6-10). For the diabetic subjects, there was no difference in the plasma AA when given in the form of AA or citrus fruit medium, which is in contrast to the normal subject supplementation with 500 mg AA/day. This could be caused by a saturation of the body AA pool with the large dosage of AA.

Fig. 5 shows a comparison of the erythrocyte sorbitol and diabetic subjects. As expected, the diabetic subjects had significantly higher baseline sorbitol levels than normal subjects (51.2 vs. 19.4 nmol/ml, respectively: P < 0.01). After supplementation, the erythrocyte sorbitol of diabetic subjects was not significantly different (P > 0.05) from the normal subjects before supplementation (28.4 and 19.4 nmol/ml, respectively). Thus, AA in the form of either AA or citrus fruit medium effectively normalised erythrocyte sorbitol of diabetic subjects. The 44.5% decrease in erythrocyte sorbitol produced by AA supplementation in the diabetic subjects is similar to the 52% reported by Malone et al.[28] after 2 weeks of therapy with sorbinil at a dosage that caused side effects in one third of the subjects in a long term study [29]. Or subjects reported no ill effects with AA.

Figure 4: Comparison of effect of 2000 mg/day ascorbic acid (ascorbate, AA) supplementation on plasma AA of normal and diabetic subjects. N Pre, AA normal subjects before supplementation; N Post, normal subjects after supplementation; D Post, diabetic subjects after supplementation.
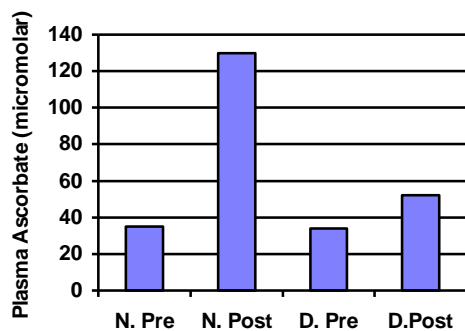
Figure 5: Comparison of effect of 2000 mg/day ascorbic acid supplementation on erythrocyte (red blood cell) sorbitol of normal and diabetic subjects. N Pre, normal subjects before supplementation; N Post, normal subjects after supplementation; D Pre, diabetic subjects before supplementation; D Post, diabetic subjects after supplementation.
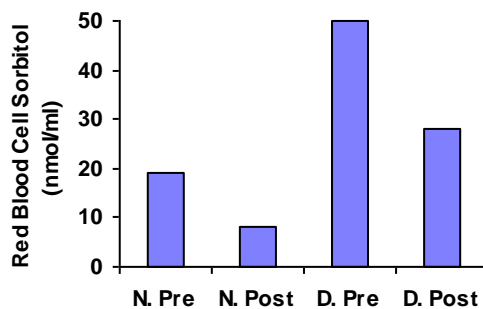
The mechanism of the effect of AA on sorbitol is unknown. AA is a well documented physiological antioxidant, and its role has been recently reviewed [30]. Previous *in*

vitro [31] and in vivo [32] studies have shown that AA is a very effective antioxidant in the lens. It has been proposed that AA functions by its physiological oxidation, which consumes NADPH indirectly through the glutothione redox couple [31]. In our laboratory, AA has been found to rapidly consume NADPH in the presence of glutathione (J.A.V. and H. W. Wang, unpublished observations). This results in less NADPH being available for sorbitol formation from glucose. Under hyperglycaemic condition, an alteration in pyridine nucleotide redox ratios produces an oxidative stress that may result in diabetic complications. Other antioxidants have been shown to reduce this oxidative stress in vitro [33].

If AA acts by a different mechanism than aldose reductase inhibitors it may prove to be synergistic with these drugs and be useful as an adjunct therapy for diabetic patients, thus allowing lower dosages of these drugs with subsequently fewer side effects. Note that in this study AA has been shown to be effective in vitro and in vivo in lowering sorbitol under normoglycemic conditions when sorbitol is ineffective [31]. Tolrestat, an aldose reductase inhibitor, has recently been shown to elevate plasma AA in diabetic rats [35]. The beneficial effect of aldose reductase inhibitors may be due at least in part to their beneficial effect on tissue AA. Studies are in progress to determine the long term effectiveness of AA and citrus fruit medium supplementation for diabetic individuals.

Acknowledgements

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