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Guinea pig ascorbate status predicts tetrahydrobiopterin plasma concentration and oxidation ratio in vivo

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ABSTRACT

Tetrahydrobiopterin (BH₄) is an essential co-factor of nitric oxide synthases and is easily oxidized to dihydrobiopterin (BH₂) which promotes endothelial nitric oxide synthase uncoupling and deleterious superoxide production. Vitamin C has been shown to improve endothelial function by different mechanisms, some involving BH₄. The hypothesis of the present study was that vitamin C status, in particular low levels, influences biopterin redox status in vivo. Like humans, the guinea pig lacks the ability to synthesize vitamin C and was therefore used as model. Seven day old animals (n = 10/group) were given a diet containing 100, 250, 500, 750, 1000, or 1500 ppm vitamin C until euthanasia at age 60–64 days. Blood samples were drawn from the heart and analyzed for ascorbate, dehydroascorbic acid (DHA), BH₄ and BH₂ by high-performance liquid chromatography. Plasma BH₄ levels were found to be significantly lower in animals fed 100 ppm vitamin C compared to all other groups (P < .05 or less). BH₂ levels were not significantly different between groups but the BH₂-to-BH₄ ratio was higher in the group fed 100 ppm vitamin C (P < .001 all cases). Significant positive correlations between BH₄ and ascorbate and between BH₂-to-BH₄ ratio and DHA were observed (P < .0001 both cases). Likewise, BH₂-to-BH₄ ratio was negatively correlated with ascorbate (P < .0001) as was BH₄ and DHA (P < .005). In conclusion, the redox status of plasma biopterins, essentially involved in vasodilation, depends on the vitamin C status in vivo. Thus, ingestion of insufficient quantities of vitamin C not only leads to vitamin C deficiency but also to increased BH₄ oxidation which may promote endothelial dysfunction.

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1. Introduction

The endothelium plays a crucial role in maintaining vascular homeostasis. A dysfunctional endothelium is observed in many diseases and conditions such as diabetes, coronary artery disease, atherosclerosis and hypertension [1]. Endothelial dysfunction (ED) is in part due to an imbalance between vasoconstricting and vasodilating substances produced by or

acting on the endothelium. ED is commonly associated with decreased bioavailability of nitric oxide (NO) due to less NO production by the endothelium and/or an increase in reaction between NO and reactive oxygen species [2]. NO is produced by nitric oxide synthase (NOS), of which there are three types: endothelial NOS (eNOS), inducible NOS and neuronal NOS. Tetrahydrobiopterin (BH₄) is an essential co-factor of the three NOSs [3]. BH₄ is easily oxidized to

Abbreviations: BH₂, dihydrobiopterin; BH₄, tetrahydrobiopterin; DHA, dehydroascorbic acid; DTE, dithioerythritol; ED, endothelial dysfunction; eNOS, endothelial nitric oxide synthase; HPLC, high performance liquid chromatography; MPA, meta-phosphoric acid; NOS, nitric oxide synthase.

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dihydrobiopterin (BH₂), which may be further oxidized to biopterin. During BH₄ deficiency, eNOS enters what is called an ‘uncoupled’ state and produces superoxide rather than NO [4], which may react to form the strong oxidant peroxynitrite. It has been found that BH₄ and BH₂ bind to eNOS with equal affinity [5,6], but whereas binding of BH₄ leads to production of NO, binding of BH₂ leads to formation of superoxide [4]. Superoxide can oxidize BH₄ directly or through the formation of peroxynitrite [7] leading to decreased production of NO and further increased production of superoxide. It has been suggested that the ratio of BH₂ to BH₄, rather than the absolute levels of BH₄ and BH₂, is the best predictor of endothelial function [5,6,8–11].

Vitamin C deficiency has been associated with increased risk of cardiovascular disease in many epidemiological studies [12] and this has prompted investigations into the potential mechanisms by which ascorbate may influence cardiovascular function. Ascorbate has been shown in *in vitro* studies to act as specific redox modulator in the NO synthesis by keeping BH₄ reduced [13–15]. Also, several mechanisms involving eNOS have been suggested through which ascorbate can potentially increase the bioavailability of NO (Fig. 1). Moreover, high doses of vitamin C has been shown to improve endothelial function in people with compromised endothelial function [1]. We therefore hypothesized that commonly observed hypovitaminosis C whilst not leading to scurvy—the ultimate clinical manifestation of vitamin C deficiency—may indirectly affect endothelial function by resulting in an unfavorable ratio of BH₄/BH₂. In the present study, we used our guinea pig model to test, if dietary vitamin C predicts the plasma concentrations and ratio of BH₄/BH₂ *in vivo*. In contrast to all other mammals, primates (including humans), bats and guinea pigs specifically lack the ability to synthesize vitamin C due to a mutation in the gene encoding for gulonolactone oxidase catalyzing the last step in the biosynthesis [16]. Thus, the guinea pig constitutes a unique and well-validated model for studying low levels of vitamin C comparable to those found in large human sub-populations [17–20].

2. Methods and materials

2.1. Materials

7,8-dihydro-L-biopterin (BH₂) and (6R)-5,6,7,8-tetrahydrobiopterin (BH₄) were from Shircks Laboratories (Jena, Switzerland). Tris(2-carboxyethyl)phosphine hydrochloride and disodium ethylenediaminetetraacetate dihydrate (Na₂-EDTA) were purchased from VWR-Bie & Berntsen A/S (Herlev, Denmark). 1,4-Dithioerythritol (DTE), and meta-phosphoric acid (MPA) were obtained from Sigma-Aldrich (Brøndby, Denmark). All other chemicals were of the highest quality available. All solutions were made in Milli-Q water.

2.2. Animals

The experiment was approved by the Animal Experiments Inspectorate under the Ministry of Food, Agriculture and Fisheries. Sixty Hartley guinea pigs (HA-SIFE150200, Charles River Laboratories, Kisslegg, Germany), 7 days of age, were marked with a subcutaneous microchip in the neck, weight stratified and randomized into six feeding groups upon arrival to our animal facility. All groups received a purified diet (Research Diets, Inc, New Brunswick, NJ, USA), the only difference being the amount of vitamin C. Ingredients are provided in Table. Diets with a final concentration of 100, 250, 500, 750, 1000, or 1500 ppm vitamin C were made from feed containing 0 ppm vitamin C (D11091304), 727.6 ppm vitamin C (D11091305) and 2128.4 ppm vitamin C (D11091306) by analysis. The animals had free access to water and hay. We have previously shown that even the lowest dose of vitamin C provided does not result in scurvy [17,21,22]. Animals were group housed in floor pens in an enriched environment at 22±2°C with a 12:12 h light–dark cycle, inspected daily by trained personnel and weighted twice weekly. At 60–64 days of age, the guinea pigs were anaesthetized by using isofluran inhalation (Isoba vet, MSD Animal Health, the Netherlands).

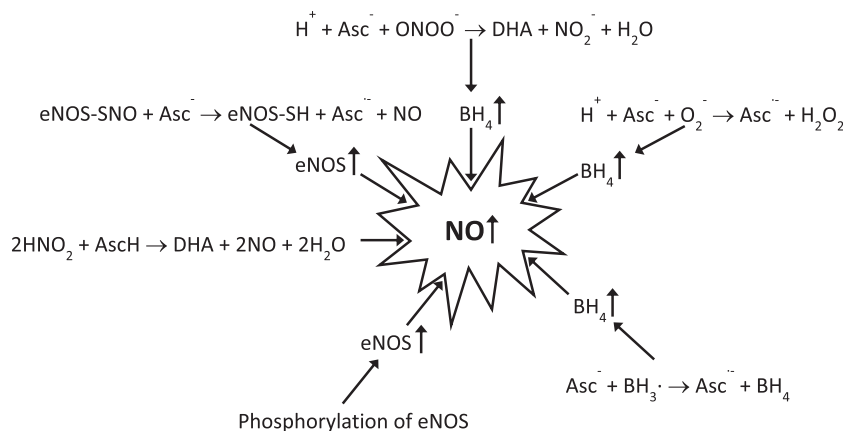


Fig. 1 – Putative mechanisms by which vitamin C may increase NO bioavailability. Vitamin C may increase eNOS activity by increasing phosphorylation [46] and decreasing S-nitrosylation [47]. Vitamin C may also increase BH₄ bioavailability by scavenging peroxynitrite [45] and superoxide [7] or by recycling one-electron oxidized BH₄ [7,41]. Ascorbic acid may also reduce nitrite to yield NO [48], though this process is likely only relevant at low pH.

Table – Ingredient composition of the diets fed to guinea pigs.

Ingredient	g			
	Diet (stock) ^a	#D11081304	#D11081305	#D11081306
Soy protein	80	80	80	80
Casein	120	120	120	120
L-Methionine	5	5	5	5
Corn starch	315	315	315	315
Maltodextrin,10	35	35	35	35
Sucrose	350	350	350	350
Cellulose, Bw200	100	100	100	100
Guar gum	25	25	25	25
Lard	20	20	20	20
Soybean oil	25	25	25	25
Mineral mix ^b	75	75	75	75
Vitamin mix ^c	10	10	10	10
Ascorbic acid	0	2.5		7.1
phosphate, L (33% active) ^d				
Choline bitartrate	2	2	2	2
Total	1162	1164.5		1169.1

^a All diets were produced by Research Diets Inc. Individual vitamin C regimes applied in the different experimental groups (100-250-500-750-1000 and 1500 ppm vitamin C) were achieved by titrating the three diets to meet the desired levels. Batch # 0 ppm vitamin C (D11091304), 700 ppm (727.6 ppm by analysis) vitamin C (D11091305), and 2000 ppm (2128.4 ppm by analysis) vitamin C (D11091306).

^b Mineral mix S20001.

^c Vitamin mix V23901.

^d Provided by Rovimix Stay-C 35.

Subsequently, a thoracotomy was performed and an intracardial blood sample obtained using a syringe with an 18G, 40 mm needle that had been flushed with 15% K₃-EDTA. Animals were finally euthanized by decapitation.

2.3. Ascorbic acid determination

Blood samples were immediately centrifuged for 2 minutes at 16000×g (4°C) and stabilized with 10% (w/v) MPA containing 2 mmol/L Na₂-EDTA as reported previously [23] and stored at –80°C until analysis. The stability of ascorbate and dehydroascorbic acid (DHA) in MPA stabilized plasma has been studied previously and found adequate in preserving the in vivo equilibrium [24,25]. Ascorbate and total vitamin C (ascorbate + DHA) following reduction were quantified by high-performance liquid chromatography (HPLC) with coulometric determination and DHA concentrations were assessed by subtraction of ascorbate from total vitamin C using uric acid as endogenous internal standard [24]. The within- and between-day coefficients of variation for the complete assay were less than 1.5% and 3.5%, respectively [26].

2.4. Biopterin determination

Blood samples for biopterin analysis were immediately added 4% DTE to yield a final concentration of 0.1% DTE. The blood sample was centrifuged for 1 min and the plasma was separated and frozen at –80°C until further analysis.

BH₂ and BH₄ were determined by HPLC modified from Hyland [27]. The HPLC system consisted of an Agilent 1100

thermostatted autosampler, an Agilent 1200 binary pump, an Agilent 1200 fluorescence detector, and an Ultimate 3000 column compartment from Dionex. BH₄ was determined using a 5011A analytical cell (ESA, Inc) with potentials of –400 mV (electrode 1) and 125 mV (electrode 2) controlled by a CouloChem II detector (ESA, Inc.)–detection was at electrode 2. BH₂ was determined by fluorescence (excitation wavelength 275 nm and emission wavelength 442 nm) after oxidation of BH₂ to the more strongly fluorescent biopterin by a conditioning cell (5021A from ESA, Inc) running at 500 mV.

The column was a Gemini C₁₈ (250×4.6 mm, 5 μm) from Phenomenex. The samples were eluted with 100% aqueous buffer containing 50 μmol/L Na₂-EDTA and 50 mmol/L ammonium acetate-acetic acid buffer at pH 4.8. The column was thermostatted at 30°C and the eluent flow was 1 mL/min.

Plasma samples were thawed immediately prior to analysis. After thawing, plasma was added 50% MPA in the ratio 9:1 (v/v). After spinning for 1 min. (16000×g), the supernatant was neutralized with 5 mol/L NaOH and analyzed immediately. Quantification was done using external standards. The within- and between-day coefficients of variation for the complete assay were less than 5 and 3.5%, respectively.

2.5. Statistical analyses

Data are presented as means ± SD unless otherwise indicated. A sample size of 10 was chosen with the purpose of identifying a 30% effect of vitamin C deficiency with a 30% SD on each average measurement and a power of 80%. Multiple regression analysis and analysis of variance were performed using Statistica (Statsoft version 9.0, Tulsa, OK, USA). In case of significance, Tukey's post hoc test was used for individual comparisons. *P* < .05 was considered statistically significant.

3. Results

As expected, plasma vitamin C was found to increase significantly with level of vitamin C in the diet (Fig. 2). However, plasma BH₄ levels were also affected by the amount of vitamin C in the diet. Thus, guinea pigs fed 100 ppm vitamin C had lower plasma levels of BH₄ (*P* < .05 or less) than animals fed higher doses of vitamin C (Fig. 3A); there were no significant differences between the other groups. BH₂ levels were unaffected by the level of vitamin C in the diet, leading to a significantly higher BH₂-to-BH₄ ratio in the group fed 100 ppm vitamin C (*P* < .0001) with no differences among the other groups (Fig. 3B).

Plasma ascorbate levels were in the range 5–100 μM, while biopterin levels ranged from around 100 nmol/L to around 400 nmol/L. Plasma levels of biopterins and ascorbate were highly correlated. As Fig. 4A shows, the BH₄ plasma level was positively correlated with total vitamin C:

$$C(\text{BH}_4)/\text{nM} = 146 + 1.24 \times C(\text{vitamin C})/\text{M} \quad P < .0001$$

BH₄ plasma concentration was also negatively correlated with the vitamin C oxidation ratio (percentage of DHA of total vitamin C) (Fig. 4B):

$$C(\text{BH}_4)/\text{nM} = 271 - 4.04 \text{ DHA} / \% \quad P < .005$$

BH₂ plasma concentration, on the other hand, was not correlated with vitamin C plasma levels. However, the ratio of BH₂ to BH₄ was. Thus, guinea pigs with high levels of total vitamin C tended to have lower BH₂-to-BH₄ ratios (Fig. 5A):

$$C(\text{BH}_2)/C(\text{BH}_4) = 0.12 - 0.00103 \times C(\text{vitamin C})/M \quad P < .0001$$

and higher percentage of DHA correlated with higher BH₂-to-BH₄ ratio (Fig. 5B):

$$C(\text{BH}_2)/C(\text{BH}_4) = -0.0055 + 0.0046 \times \text{DHA}/\% \quad P < .0001.$$

4. Discussion

A considerable epidemiological literature has found that vitamin C deficiency in humans is consistently associated with increased risk of developing cardiovascular disease and stroke (for recent studies see [28–31]). In contrast, all major intervention studies have been unable to demonstrate an effect of vitamin C supplementation on cardiovascular risk (for recent studies see [32–35]). However, as pointed out by us elsewhere, none of the latter studies has unfortunately recruited and thus studied the effect of supplementation in deficient individuals which is of major importance considering the particular non-linear saturation kinetics of vitamin C [20,12,36]. Thus, properly performed animal model studies of vitamin C deficiency are indeed warranted. In the present study, we wanted to investigate the *in vivo* relationship between plasma biopterin redox status, vitamin C ingestion and plasma vitamin C using guinea pigs as a model as a negative effect of vitamin C deficiency on biopterin redox status *in vivo* may constitute an indirect rationale underlying the association between poor vitamin C status and increased risk of heart disease observed in humans.

Some smaller intervention studies have shown a beneficial—but typically transient—effect of vitamin C on endothelial function in people suffering from various conditions such as

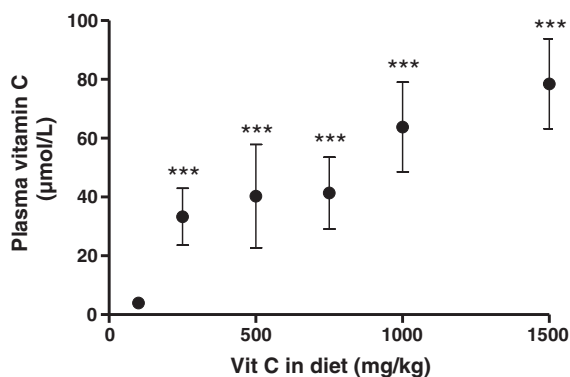


Fig. 2 – Plasma vitamin C as a function of vitamin C in diet. Guinea pigs (n = 10 in each group) were exposed to different vitamin C concentrations for two months. Plasma vitamin C was significantly lower in the group fed 100 ppm vitamin C compared to all other groups. Data are presented as means ± SD. ***P < .001.

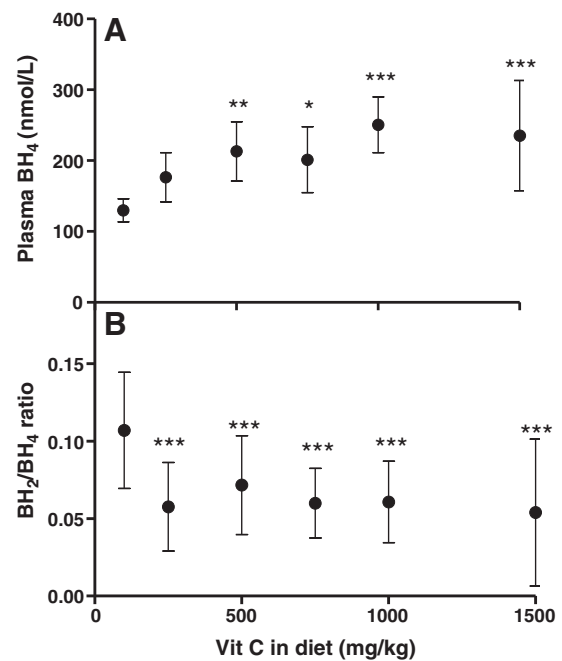


Fig. 3 – Plasma BH₄ level and BH₂-to-BH₄ ratio as a function of vitamin C in the diet. Guinea pigs (n = 10 in each group) were exposed to different vitamin C concentrations for two months. BH₄ levels (A) were significantly lower and BH₂-to-BH₄ ratio (B) was significantly higher of the 100 ppm group compared to the other groups. Data are presented as means ± SD. *P < .05, **P < .01, ***P < .001.

diabetes, coronary artery disease, hypertension, and inflammation, while other studies found no effect of such intervention (for a review see [1]). Numerous possible mechanisms behind a putative effect of vitamin C on endothelial function have been suggested, e.g. decrease in low-density lipoprotein oxidation, scavenging of superoxide, release of nitric oxide from S-nitrosothiols, reduction of nitrite to nitric oxide, and activation of either eNOS or smooth muscle guanylate cyclase [37]. Of particular relevance to our observations are studies looking at the relationship between vitamin C and eNOS activity, since BH₄ is a necessary co-factor of eNOS and plasma levels of vitamin C and redox status of biopterins are linked. Vitamin C could exert its positive effect on eNOS activity directly through regulation of eNOS activity or indirectly by increasing the BH₄ bioavailability. Several potential mechanisms involving eNOS have been suggested through which ascorbate could increase the bioavailability of NO. In a number of studies, it was found that incubation of endothelial cells in the presence of vitamin C increased intracellular BH₄ levels [13–15], whereas another study found only a non-significant increase of intracellular BH₄ when endothelial cells were incubated with vitamin C alone but a marked 176% increase when cells were treated with E. coli endotoxin and vitamin C together [38]. Likewise, incubation of mouse macrophages with vitamin C was found to increase intracellular levels of BH₄ [39]. Based on *in vitro* experiments it has been suggested that vitamin C exerted its positive effect on BH₄ levels by preventing oxidation of BH₄ or by recycling one-electron oxidized BH₄ [7,14,40,41]. One way that ascorbate

could prevent oxidation of BH₄ is by scavenging reactive oxygen species before they react with BH₄, which is very easily oxidized. Several reactive oxygen species exist but of particular relevance to NO bioavailability is superoxide and peroxynitrite. Superoxide reacts with both ascorbate and BH₄ with a rate constant of 3 to 4·10⁵ mol/L⁻¹ s⁻¹ [42,43]. The much higher plasma concentration of ascorbate compared to BH₄ means that ascorbate at least theoretically is capable of protecting BH₄ from oxidation by superoxide. However, superoxide reacts much faster with NO forming peroxynitrite than with either ascorbate or BH₄. Thus, the typically micromolar plasma concentrations of ascorbate have been found inadequate in preventing superoxide from reacting with NO. In fact, ascorbate concentrations of 10 mmol/L are needed to compete with NO for superoxide [44]. In contrast, in vitro experiments with endothelial cells, ascorbate concentrations were found in the low millimolar range [45] suggesting that ascorbate would be able to scavenge at least part of the superoxide generated intracellularly. Moreover, although peroxynitrite reacts 10 times faster with BH₄ than with ascorbate [7], the 1000-fold higher concentration of ascorbate compared to BH₄ in human plasma suggests that peroxynitrite may react with ascorbate over BH₄ in vivo. Another possible mechanism by which ascorbate could affect the bioavailability of BH₄ is through regulation of the synthesis of BH₄. BH₄ is synthesized in vivo by two mechanisms referred to as de novo synthesis and the salvage pathway, respectively [3]. It was

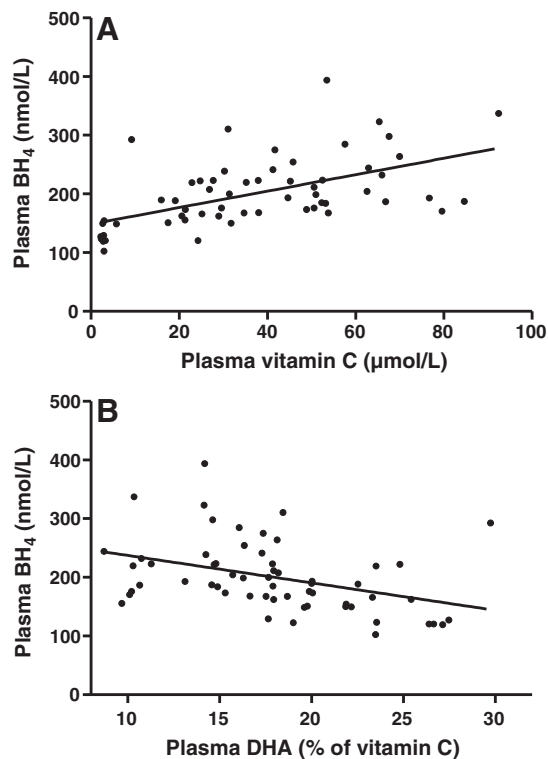


Fig. 4 – Correlation between BH₄ and vitamin C. Data from all animals (n=60) were included in the analysis. Plasma BH₄ levels were highly ($P < .0001$) positively correlated with plasma vitamin C (A) and highly ($P < .0001$) negatively correlated with DHA (B).

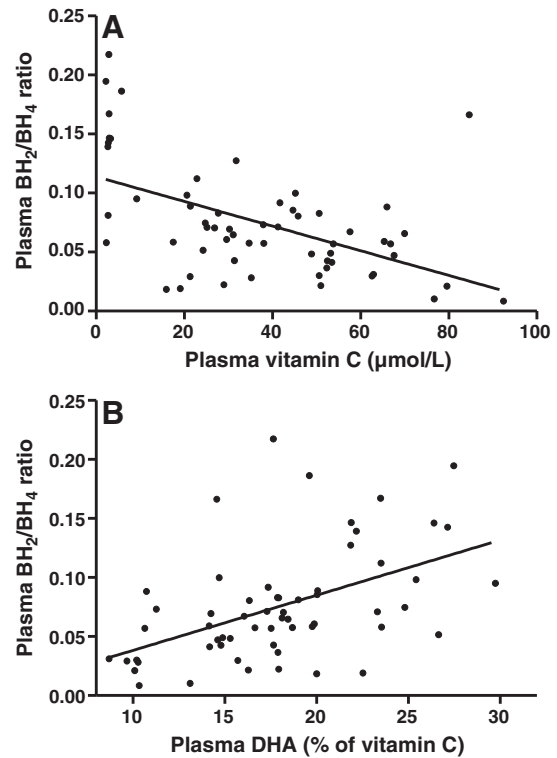


Fig. 5 – Correlation between biopterin ratio and vitamin C. Data from all animals (n = 60) were included in the analysis. Plasma BH₂-to-BH₄ ratio is highly ($P < .0001$) negatively correlated with plasma vitamin C (A) and highly ($P < .0001$) positively correlated with DHA (B).

found that ascorbate had no effect on the expression or activity of the rate-limiting enzyme (GTPCH-I) in the de novo synthesis of BH₄ [14] and, so far, no effect of ascorbate on the salvage pathway has been reported.

Ascorbate could also affect the NO bioavailability by directly influencing the activity of eNOS. In a very recent report, it was shown that vitamin C increases phosphorylation of eNOS-Ser¹¹⁷⁷ and decreases phosphorylation of eNOS-Thr⁴⁹⁵, which is indicative of increased eNOS activity [46]. Besides phosphorylation, eNOS activity is also regulated by S-nitrosylation of its cysteines, whereby S-nitrosylation leads to reduced activity. It has been shown that ascorbate was able to denitrosylate eNOS, hereby increasing eNOS activity [47]. Finally, ascorbic acid could also increase NO bioavailability directly by reducing nitrite—formed by oxidation of NO—to NO, a process utilized in the curing of meat. However, this reaction requires an acidic environment, such as that found in the stomach, to proceed at an appreciable rate. Thus, at pH 3.63 the reaction was found to proceed at an appreciable rate but was found to be much slower at pH 5.49 [48]. Consequently, in the vasculature, enzymatic reduction of nitrite to NO is more likely than reduction by ascorbate [49].

Few studies have looked at the effect of vitamin C on in vivo biopterin levels. d'Uscio et al observed that long-term vitamin C supplementation increased aortic BH₄ levels and decreased BH₂-to-BH₄ levels in wild-type mice, whereas BH₂ levels were unaffected [50], findings that are in-line with our observations although their study was conducted in a species

capable of synthesizing vitamin C. Likewise, it was found that BH₄ levels were decreased and BH₂ levels were increased in diabetic rat hearts compared to control rat hearts and that vitamin C could alleviate these changes [51]. In a study in guinea pigs, neutrophils isolated from scorbutic guinea pigs were found to contain less BH₄ than the neutrophils from control animals [52].

In the present study, the BH₄ plasma level ranged from 100 to 400 nmol/L with a mean of around 200 nmol/L. BH₂ levels were typically between 5 and 30 nmol/L with an average of 15 nmol/L. The biopterin plasma levels have, to the best of our knowledge, not previously been measured in guinea pigs. The level of plasma BH₄ in our guinea pigs is an order of magnitude higher than that found in humans, which in various studies have been found to range from a few nmol/L up to 35 nmol/L [8,53–84]. BH₄ in human plasma typically comprises around 50% to 80% of total biopterins whereas in guinea pigs, more than 90% is on the reduced form. Compared to other animal species, guinea pig biopterin plasma levels are comparable to those found in studies using mice and rats [57,66,83,85–91], whereas larger animals such as dogs, cats, and monkeys have biopterin levels more similar to those found in humans [65,66,83,92]. Thus, one limitation of our study is that the impact of vitamin C deficiency on biopterin status may not translate between guinea pigs and humans as only the vitamin C level is similar between the two species while guinea pig biopterin levels are higher. On the other hand one might speculate that the impact of vitamin C deficiency could be even larger in humans as the low biopterin levels presumably are more prone to oxidation.

Our studies show that high plasma vitamin C levels correlate with high BH₄ plasma levels and a low BH₂-to-BH₄ ratio in guinea pigs. However, plasma concentration of vitamin C is not the only predictor of BH₄ plasma levels and BH₂-to-BH₄ ratio: DHA, a measure of oxidative stress, also correlates with BH₄ and BH₂-to-BH₄ ratio. Thus, a high proportion of DHA was found to correlate with a high BH₂-to-BH₄ ratio, also indicating a state of increased oxidative stress.

In conclusion, we can accept our hypothesis that biopterin redox status depends on plasma vitamin C levels in that low levels of plasma vitamin C leads to a higher BH₂-to-BH₄ ratio. Thus, our data provide in vivo support for a relationship between ascorbate status and biopterin bioavailability and may therefore indirectly explain clinical observations showing a negative impact of vitamin C deficiency on cardiovascular health since a high BH₂-to-BH₄ ratio has been linked to endothelial dysfunction in several studies [5,6,8–11]. However, the human relevance of the ascorbate-dependent maintenance of a reduced BH₄ pool clearly needs to be confirmed in controlled clinical studies.

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