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High dietary fat and cholesterol exacerbates chronic vitamin C deficiency in guinea pigs

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Abstract

Vitamin C deficiency – or hypovitaminosis C defined as a plasma concentration below 23 μM – is estimated to affect hundreds of millions of people in the Western world, in particular subpopulations of low socio-economic status that tend to eat diets of poor nutritional value. Recent studies by us have shown that vitamin C deficiency may result in impaired brain development. Thus, the aim of the present study was to investigate if a poor diet high in fat and cholesterol affects the vitamin C status of guinea pigs kept on either sufficient or deficient levels of dietary ascorbate (Asc) for up to 6 months with particular emphasis on the brain. The present results show that a high-fat and cholesterol diet significantly decreased the vitamin C concentrations in the brain, irrespective of the vitamin C status of the animal ($P<0{\cdot}001$). The brain Asc oxidation ratio only depended on vitamin C status ($P<0{\cdot}0001$) and not on the dietary lipid content. In plasma, the levels of Asc significantly decreased when vitamin C in the diet was low or when the fat/cholesterol content was high ($P<0{\cdot}0001$ for both). The Asc oxidation ratio increased both with low vitamin C and with high fat and cholesterol content ($P<0{\cdot}0001$ for both). We show here for the first time that vitamin C homoeostasis of brain is affected by a diet rich in fat and cholesterol. The present findings suggest that this type of diet increases the turnover of Asc; hence, individuals consuming high-lipid diets may be at increased risk of vitamin C deficiency.

Key words: **Vitamin C deficiency: High-fat diet: Brain**

For many years, the sole accepted clinical consequence of vitamin C deficiency has been an increased risk of developing the rare but potentially mortal disease scurvy. In contrast, many epidemiological studies have found an association between chronic vitamin C deficiency and increased risk of developing a range of age-associated conditions such as CVD, type 2 diabetes and cancer[1–5]. More recently, vitamin C deficiency has been linked to impaired brain development and cognitive deficits in experimental animal studies[6–8], consequences that may also be relevant in humans[9]. The intracellular concentration of ascorbate (Asc) in neurons is up to tenfold higher than in reference tissues such as the liver. Also the brain is capable of retaining and recycling vitamin C during states of deficiency, leaving it to be the last of tissues to become depleted[6,10,11]. The functions of vitamin C in the brain are numerous[9]. Not only is Asc directly involved in the biosynthesis of neurotransmitters and as a modulator of neurotransmission, but it is also an essential factor for neural maturation[12–14]. Hence, during vitamin C deficiency, functional consequences are expected to occur, and recent *in vivo* data support this hypothesis by finding impaired memory, decreased number of neurons in hippocampus, as well as sensorimotor deficits in vitamin C-deficient mice and guinea pigs[7,8,15].

In humans, the subpopulations with the lowest intake of vitamin C – and therefore the highest risk of developing deficiency – are people of low socio-economic status who tend to eat a poor diet low in fruits and vegetables[16–18]. However, an unhealthy diet not only lacks fruits and vegetables, i.e. the primary dietary sources of vitamin C, but also tends to contain high levels of TAG and cholesterol. In fact, blood levels of cholesterol and TAG are apparently affected by the concentration of vitamin C since both the activity of the rate-limiting enzyme, 7α-hydroxylase,[19] in the production of bile acids from cholesterol and the fatty acid transport rate via the carnitine transport system show vitamin C dependency[20]. This association has been confirmed by reports showing increased plasma concentrations of cholesterol and fatty

Abbreviations: Asc, ascorbate; DHA, dehydroascorbic acid.

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acids during vitamin C deficiency[20–22]. In contrast to these previous studies, our interest has been vitamin C status *per se*, and in the present paper, we report the effect of a poor diet rich in fat on the vitamin C homoeostasis of the brain. For the purpose of combining vitamin C deficiency with diet-induced hypercholesterolaemia, we have used guinea pigs as they are considered validated models for the human state of both conditions[23,24].

Experimental methods

Materials

All chemicals were of the highest quality available. Specifically, Asc, dehydroascorbic acid (DHA), GSH, dansyl chloride, α- and γ-tocopherol, and *t*-butyl hydroperoxide were obtained from Fluka (Milwaukee, IL, USA). *meta*-Phosphoric acid and disodium EDTA were obtained from Merck (Darmstadt, Germany).

Animals

The study was approved by the Danish Animal Experimentation Inspectorate under the Ministry of Justice. Female Dunkin Hartley Guinea pigs (12 weeks of age) were obtained from HP-Lidkjöbing Kaninfarm (Lidkjöbing, Sweden). Upon arrival at the University animal facility, the animals were left for 2 weeks of acclimatisation after which each animal was tagged with a 12 mm microchip inserted subcutaneously in the neck for identification (PET-Id; Danworth Farm, West Sussex, UK). The animals were housed in floor bins allowing approximately 0·2m^2 per animal and equipped with toys and hiding places to ensure a proper environment. Animals were attended daily by trained staff, checked regularly for clinical signs of disease and weighed every 4 weeks. Also the animals were routinely observed for weight loss, paralysis of the rear legs and haemorrhages in order to rule out the presence of scurvy symptoms. Before the experiment, the animals were randomised into four weight-stratified groups each receiving one of four experimental diets given below. The guinea pigs were followed for up to 6 months under the above conditions with monthly blood samplings (4 × 70 μl) drawn by puncturing of *v. saphena lateralis*. After 1, 2, 3 and 6 months, guinea pigs were anaesthetised using 0·08 ml/100 g body weight of supplemented zoletil solution (0·48 mg/ml Zoletil 50 vet. (Virbac S.A., Carros Cedex, France)), 2 mg/ml xylazine (Narcoxyl, Intervet Int., Boxmeer, Holland) and 0·5 mg/ml butorphanol tartrate (Torbugesic, ScanVet, Fredensborg, Denmark), in isotonic NaCl). Animals were briefly supplemented with CO_2 inhalation (70 % CO_2:30 % O_2) until disappearance of (interdigital and palpebral) reflexes, immediately after which thoracotomy was performed followed by intracardial blood sampling. A 5 ml syringe with an 18G needle previously flushed with 15 % tripotassium EDTA was used. Ultimately, the animals were euthanised by exsanguination while still anaesthetised. The blood samples were carefully handled to avoid haemolysis and were immediately processed as described below. Brains were removed and immediately frozen to −80°C until further analysis. Liver was used as a reference tissue and also stored at −80°C.

Diets

The animals were fed diets that combined adequate/insufficient amounts of vitamin C with low/high amounts of fat and cholesterol in a 2 × 2 factorial design, i.e. high C/low fat (fifteen animals), low C/low fat (sixteen animals), high C/high fat (thirty-two animals) and low C/high fat (thirty-four animals). The diets consist of quality-controlled conventional cereal-based formulations (low fat; product codes 829 415 (low C) and 829 427 (high C)) or synthetic foods (high fat; product codes 824 163 (low C) and 824 158 (high C); Special Diet Services, Dietex, Witham, UK), and the vitamin C levels in the diets were chosen in order to reflect the plasma concentrations found in human subjects receiving adequate and inadequate vitamin C, respectively, but without resulting in scurvy symptoms. By analysis the 'high C' diets contained 691 mg per kg feed of vitamin C (as a stable phosphate derivative, Stay-C; Roche, Basel, Switzerland) and the deficient ('low C') diets contained 100 mg of vitamin C per kg feed. The exact composition of the low-fat and the high-fat diets can be seen in Table 1. The high-fat diet was formulated to resemble the diet given in the study by Cos *et al.*[25].

Biochemical analysis

Blood samples were immediately centrifuged (2000 *g* for 5 min at 4°C). For total Asc (total Asc = Asc + DHA measured after reduction with Tris (2-carboxyethyl) phosphine hydrochloride) and DHA analysis, one 50 μl aliquot of plasma was acidified with an equal volume of 10 % *meta*-phosphoric acid containing 2 mM-EDTA, briefly vortex mixed and centrifuged (16 000 *g* for 1 min), and the

Table 1. Composition of experimental diets

Low-fat diet		High-fat diet	
Component	% (w/w)	Component	% (w/w)
Wheatfeed bulk	34·5	Maize starch	33
Barley	19	Soya protein	23
Dehydrated grass	15	Cellulose	10
Extracted linseed	11	Sucrose	10
Oat	9	Palm oil	6·3
Soya and potato protein	5	Safflower oil	5·6
Mineral and vitamin mix*	4·2	Olive oil	3
Maize and wheat gluten	2·3	Guar gum	2·5
		Mineral and vitamin mix*	5
		Cholesterol	0·33

* 100 mg/kg vitamin C in the 'low C' diets and 691 mg/kg vitamin C in the 'high C' diets.

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supernatant was frozen at −80°C. The analytical procedure using HPLC with coulometric detection has been described in detail elsewhere[26,27]. The remaining plasma was stored neat at −80°C. α-and γ-Tocopherol concentrations were measured in plasma samples by HPLC with amperometric detection following hexane extraction as described by Sattler *et al.*[28].Total GSH level of brain was measured after derivatisation with dansyl chloride by HPLC with fluorescence detection[29].

Plasma total cholesterol and TAG were measured spectrophotometrically by use of commercial kits (CHOD-PAP and GPO-PAP, respectively; Roche, Hvidovre, Denmark).

Statistics

Statistical analysis was carried out by using Statistical Analysis Systems software package version 9.1 (SAS Institute, Cary, NC, USA). Plasma total Asc and Asc oxidation ratio (%DHA of total Asc) over time were logarithm-transformed and analysed statistically using a linear mixed model with a two-way interaction between diet combinations (vitamin C and fat/cholesterol) and observation times as the fixed effects. Random effects were used to account for variation between guinea pigs. Estimation and hypothesis testing were carried out using the restricted maximum likelihood method in SAS PROC MIXED. Brain 'total Asc' and '%DHA' were analysed over time by a three-way ANOVA, with time, fat and vitamin C as factors. All the data in Table 2 were analysed by a two-way ANOVA with fat and vitamin C in diet as factors. A P value $<0{\cdot}05$ was considered statistically significant.

Results

Plasma ascorbate and ascorbate oxidation ratio

The plasma concentrations of Asc became stable after 2 weeks on the respective diets (Fig. 1(a)). The plasma Asc concentration of the control animals (high C/low fat) did not change significantly during the study, whereas high C/high-fat animals dropped to about 70 % of the initial level. The plasma Asc concentrations of the low C/low-fat animals decreased to approximately 15 % of the initial level and even significantly lower (approximately 8 %) in the group receiving the low C/high-fat diet. The statistical analysis revealed that the plasma concentration of Asc depended on both vitamin C in the diet and on the fat/cholesterol content ($P<0{\cdot}0001$ in both cases). When no fat/cholesterol was added to the diets, the levels of vitamin C were about 50 % higher than those observed in the corresponding high-fat diets regardless of the vitamin C content.

In the first few weeks of the study, there were marked changes in the levels of the Asc oxidation ratio (Fig. 1(b)). The DHA of the sufficiently supplied animals decreased to levels below 10 %, with the high C/high-fat animals having

Table 2. Biochemical measurements in plasma, brain and liver in guinea pigs subjected to four different 6-month dietary regimens
(Mean values and standard deviations)

Vitamin C in diet…	High (*n* 7)		Low (*n* 8)		High (*n* 7)		Low (*n* 9)		Two-way ANOVA	
Fat/cholesterol in diet…	Low (*n* 7)		Low (*n* 8)		High (*n* 7)		High (*n* 9)		Effect of fat	Effect of vitamin C
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	*P*	*P*
Plasma										
Total ascorbate (μM)	43·2	9·8	6·8	3·4	31·6	16·1	4·3	2·0	0·0527	<0·0001
Dehydroascorbic acid (μM)	0·70	0·32	0·38	0·55	0·45		0·47	0·26	NS	NS
Dehydroascorbic acid (% of total ascorbate)	1·7	0·81	5·5	6·0	2·0	2·0	12·0	7·4	0·0542	0·001
Cholesterol (mM)	0·80	0·15	0·84	0·17	3·8	0·67	4·6	1·9	<0·0001	NS
TAG (mM)	0·98	0·40	1·1	0·22	1·0	0·47	1·5	1·5	NS	NS
α-Tocopherol (μM)	1·7	0·61	2·2	0·53	14·2	2·8	15·0	8·5	<0·0001	NS
γ-Tocopherol (μM)	0·23	0·12	0·12	0·07	0·95	0·42	0·90	0·55	<0·0001	NS
Brain										
Total ascorbate (nmol/g tissue)	1221	66	595	265	1039	72	357	173	0·0013	<0·0001
Dehydroascorbic acid (nmol/g tissue)	62·3	65·1	49·6	40·9	31·9	17·9	26·9	19·0	0·0645	NS
Dehydroascorbic acid (% of total ascorbate)	5·0	5·2	9·7	7·6	3·0	1·6	9·2	6·3	NS	0·0121
GSH (nmol/g tissue)	1567	121	1596	115	1323	119	1405	119	<0·0001	NS
Liver										
Total ascorbate (nmol/g tissue)	1348	178	340	162	878	225	193	49	<0·0001*	<0·0001*

* Data were logarithm transformed before statistical analysis.

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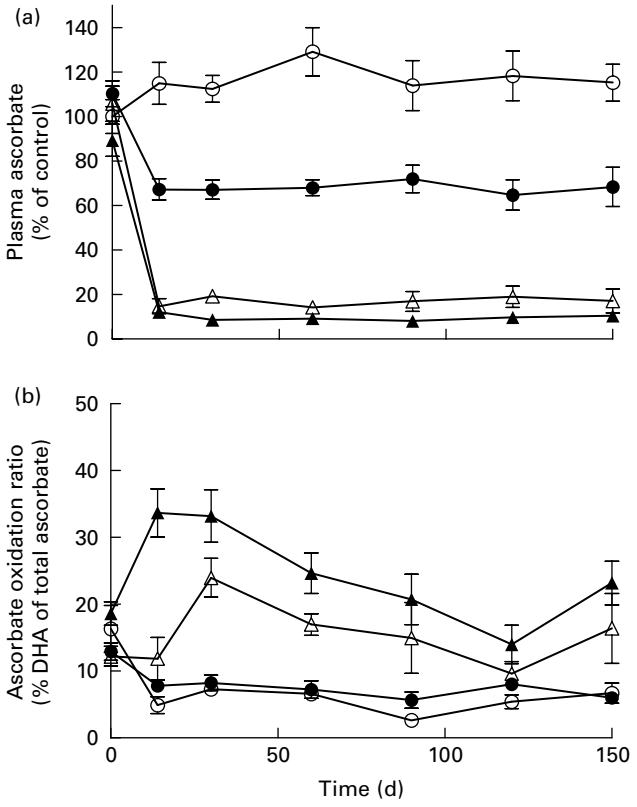

Fig. 1. Vitamin C status in the plasma of guinea pigs fed the four different diets. Plasma ascorbate (Asc) is expressed as a percentage of the initial Asc concentration in control animals with high C/low-fat diet (a). Asc oxidation ratio in plasma is expressed as % dehydroascorbic acid of total ascorbate (b). Value for each data point is *n* 7 or more. Values are represented as means with their standard errors; if error bars are not visible, it indicates that they are smaller than dot size. Dietary regimens: high C/low fat (○), high C/high fat (●), low C/low fat (△) and low C/high fat (▲). Three-way ANOVA using time, dietary fat and dietary Asc as factors revealed significant effects of all on both plasma Asc and plasma Asc oxidation ratio ($P<0{\cdot}001$ in all cases).

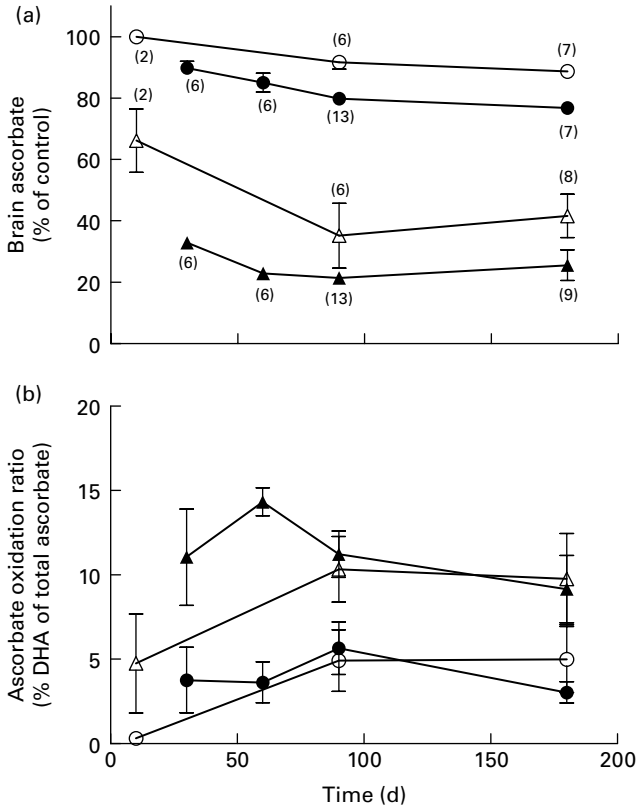

Fig. 2. Vitamin C status in the brains of guinea pigs fed the four different diets. Brain ascorbate (Asc) is expressed as a percentage of the initial Asc concentration in control animals with high C/low-fat diet (a). Asc oxidation ratio in brain is expressed as % dehydroascorbic acid (DHA) of total Asc (b). Value for each data point is given in parentheses in panel A. Values are represented as means with their standard errors; if error bars are not visible, it indicates that they are smaller than dot size. Dietary regimens: high C/low fat (○), high C/high fat (●), low C/low fat (△) and low C/high fat (▲). Three-way ANOVA with time, dietary content of Asc and fat revealed that brain Asc was significantly decreased by low C ($P<0{\cdot}001$) and high fat ($P<0{\cdot}001$), whereas brain Asc oxidation ratio was only significantly decreased by low C ($P<0{\cdot}0001$) and not high fat.

small but significantly higher levels than the high C/low-fat group. In contrast, the Asc oxidation ratio of the deficient animals increased twofold with the low-fat diet and even threefold with the high-fat diet. After the first month, the levels gradually declined but the difference persisted. In the animals fed the sufficient vitamin C diets (high C/low or high fat), Asc oxidation ratio levels remained largely unchanged. Both vitamin C and fat/cholesterol content of the diet significantly affected Asc oxidation ratio ($P<0{\cdot}0001$ in both cases). Yet the relative change was larger (54%) when the vitamin C status of the animals was deficient as compared with when they were fed sufficient vitamin C (23%).

Brain ascorbate and ascorbate oxidation ratio

The brain tissue levels of Asc in the sufficiently supplied animals remained relatively stable at about 1200 nmol/g tissue and at 1000 nmol/g tissue for groups with low- and high-fat diets, respectively (Table 2 and Fig. 2(a)). After 6 months, the Asc level was 12% lower in the high fat as compared with low fat-fed animals. When the animals were kept on the diets deficient in vitamin C, the levels of Asc in the brain decreased over time during the first 90 d of the study, after which they stabilised at much lower levels than those observed for the vitamin C-sufficient diets (37 and 23% compared with controls for groups with low and high in fat, respectively; Fig. 2(a)). The pattern observed in the brain was thus largely reflected by the plasma concentrations, namely that the addition of fat and cholesterol to the respective diets significantly reduced the levels of vitamin C. A three-way ANOVA analysis revealed that both vitamin C ($P<0{\cdot}001$) and fat ($P<0{\cdot}001$) in the diet affected the vitamin C status of the brain. The Asc oxidation ratio levels (Fig. 2(b)) were higher in animals fed diets deficient in vitamin C (approximately 10%) compared with those on vitamin C-adequate diets (approximately 5%) ($P<0{\cdot}0001$). The ability of the brain to keep its Asc pool reduced was hence significantly dependent on the amount of vitamin C in the diet, whereas

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fat and cholesterol in the diet did not significantly affect the Asc oxidation ratio.

In the statistical models for both total ascorbic acid and %DHA, no interactions with vitamin C in diet, fat/cholesterol in diet or time turned out to be significant, and they were thus omitted from the model.

Plasma and tissue antioxidants, cholesterol and TAG

Table 2 shows the biochemical data from plasma, brain and liver tissue after 180 d on the respective four diets. For all the data in Table 2, effects of vitamin C and fat were tested by the two-way ANOVA analysis. As the data in Table 2 only reflect single time points in contrast to the curves in Figs. 1 and 2, the lower power of these resulted in merely near-significant tendencies for the effect of fat in the diets in some cases (Table 2).

Cholesterol increased from 0·82 to 4·29 mM when it was added to the diet indicating hypercholesterolaemia. The cholesterol level was not affected by the vitamin C content of the diet. Surprisingly, the levels of TAG were unaltered in between groups. Both α- and γ-tocopherols increased several folds when fat and cholesterol were added to the diets (Table 2). The amount of vitamin C in the diet had no effect on the plasma tocopherol levels.

After 180 d, GSH levels in brain tissue did not alter when vitamin C was insufficient in the diet but remained 1600 nmol/g tissue, whereas with fat and cholesterol in the diet, the levels decreased slightly but significantly to 1400 nmol/g tissue.

The liver was used as a reference tissue. In the liver, the levels of total ascorbic acid decreased when the vitamin C in the diet was marginally insufficient (from 1348 to 340 nmol/g tissue). High dietary fat content caused the levels to significantly decrease even further (Table 2).

Discussion

When studying the long-term effects of marginal vitamin C deficiency, it is of uttermost importance to ensure that clinical scurvy does not evolve. During scurvy, vast metabolic changes take place, which might cause inconsistent experimental findings, hence obstructing the conclusions made from the study[30]. As described in the experimental methods section, the guinea pigs were examined on a regular basis for symptoms of scurvy, and no animals were found to be affected.

In the present study, we examined the effect of high dietary fat and cholesterol on the vitamin C homoeostasis of brain. In spite of the effective systems maintaining high brain concentrations of vitamin C[6], the addition of high fat and cholesterol to the guinea pig diets caused the levels of vitamin C to significantly decrease irrespective of the vitamin C content of the diet. Also the Asc oxidation ratio was affected by the different diets. In plasma, vitamin C deficiency as well as high fat/cholesterol increased the ratio of oxidised to reduced vitamin C, whereas oxidation ratios in the brain were affected only by vitamin C deficiency. In agreement with its use as a biomarker of redox imbalance, an increased oxidation ratio suggests that the capacity to recycle DHA to Asc is overwhelmed by the rate of Asc oxidation[31–33]. Previously, guinea pig studies have found that diets high in cholesterol caused the plasma and/or hepatic levels of vitamin C to decrease[34–40]. The specific mechanism for this is still unknown, but it has been suggested that hypercholesterolaemia itself has the ability to increase systemic oxidative stress[41,42]. The lipid metabolism in guinea pigs has been found to resemble that of humans. It has also been reported that production of reactive oxygen species increases selectively in adipose tissue of obese mice most likely through augmented expression of NADPH oxidase and decreased expression of antioxidative enzymes, supporting a link between high fat and oxidative stress[43]. Moreover, there is the possibility that the high amount of cholesterol and fat in the high-fat diet has interfered with the absorption of vitamin C in the intestines as dietary factors have been hypothesised to affect the function of the sodium-dependent vitamin C transporter 1, which is responsible for vitamin C uptake in the intestines[44]. As regarding factors such as cholesterol synthesis, lipoprotein content and composition, activity of enzymes involved in lipoprotein metabolism etc.[23,45,46], it is possible that hypercholesterolaemia could have implications for the severity of vitamin C deficiency in humans as well.

In the present study, the GSH level of brain was significantly decreased when animals were subjected to a high-fat diet (Table 2), which further supports the hypothesis that hypercholesterolaemia might increase systemic oxidative stress and burden the antioxidant defence system. The levels of the vitamin E isoforms (α- and γ-tocopherol) were also affected by the fat content of diet. They both increased substantially when the diet was high in fat, i.e. contained a fat mixture made from vegetable oils, which are well-known dietary sources of vitamin E[47]. *In vivo* experiments in other species have confirmed a rise in vitamin E levels as a consequence of a high-fat/high-cholesterol diet[48]. The most likely reason for this increase in vitamin E is probably the dietary composition itself rather than a change in redox balance. Also, there is the possibility that inflammation in the gastrointestinal tract due to incompatibility with the high amounts of fat and cholesterol in the diet could ultimately lead to lipolysis and hence an increased release of vitamin E[49].

Yet the vitamin C status of the animals did not significantly interfere with GSH or vitamin E status. We have previously reported that brain antioxidants other than vitamin C (i.e. GSH, superoxide dismutase and vitamin E) in guinea pigs were unaffected by vitamin C deficiency or even vitamin C depletion[6,7]. Even though there is some *in vivo* evidence that vitamin C, glutathione and vitamin E status are linked to one another[50–52], there have been

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several reports on vitamin C deficiency showing no effect on vitamin E and glutathione levels[53–58]. Concerning GSH, this redox modulator is endogenously produced and hence may become compensatorily up-regulated when oxidative stress is increased as a result of vitamin C depletion. Vitamin E levels could also be affected by a severe hypovitaminosis C, as vitamin C is believed to play a role in keeping vitamin E reduced[59,60], yet this does not seem to always affect the actual amounts of vitamin E isoforms.

These observations together with the present findings could indicate that the oxidative stress exerted by an increased intake of dietary fat and cholesterol affects the antioxidant defence in the brain in a different manner than vitamin C deficiency alone.

The kinetics for the vitamin C deficiency to develop was different between plasma and brain tissue. The plasma reached a steady-state concentration within a few weeks, whereas the stabilisation of brain total Asc took longer. This is in agreement with the general belief that the brain is especially sensitive to lack of vitamin C and hence has developed extended ability to preserve the vitamin C pool[6,61].

Another lifestyle-related factor that is known to negatively affect vitamin C status is smoking. It has successively been documented that smokers have significantly lower levels of vitamin C. This lower vitamin C status in smokers has been shown to be partly due to an increased turnover as a consequence of increased systemic oxidative stress of the smoke *per se* but also partly due to a generally poorer diet with less fruits and vegetables consumed by smokers compared with non-smokers[62–66]. These findings have resulted in recognition of the fact that smokers may require a higher intake of vitamin C compared to healthy controls in order to keep the plasma levels of this vitamin at a sufficient level. In agreement with this rationale, special recommendations were included in the latest revision of the dietary reference intakes in which the RDA for smokers was increased with 35 mg vitamin C/d compared with non-smokers as compensation[67,68]. The present findings suggest that the dietary component of the vitamin C depletion observed in smokers may not only be attributed to a decreased consumption of dietary sources of vitamin C but also be attributed to a increased consumption of fat. Indeed, it has been reported that smokers tend to eat more fat through the diet resulting in higher levels of serum cholesterol, TAG and LDL:HDL ratios[69].

As the present study design does not allow us to distinguish between decreased absorption fraction and increased turnover of vitamin C, we can only report the decreased relative bioavailability in high-fat-fed animals compared to those consuming a low-fat diet. Also, it is not known whether the impact of fat and cholesterol on the plasma vitamin C status is comparable between the non-producing species. Thus, future studies are needed to clarify similarities and possible differences between the vitamin C homoeostasis of guinea pigs and humans. However, if the fat-induced vitamin C depletion observed in the present study also occurs in humans, it may have implications for future revisions of the dietary reference intakes suggesting that also people with a diet rich in fat should have a higher RDA of vitamin C.

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