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Chronic Vitamin C Deficiency does not Accelerate Oxidative Stress in Ageing Brains of Guinea Pigs

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Abstract: Increased oxidative stress in the brain has consistently been implied in ageing and in several degenerative brain disorders. Acting as a pivotal antioxidant in the brain, vitamin C is preferentially retained during deficiency and may play an essential role in neuroprotection during ageing. Thus, a lack of vitamin C could be associated with an increase in redox imbalance in the ageing brain. The present study compared oxidative stress of ageing to that of a long-term non-scorbutic vitamin C deficiency in guinea pigs. Adults (3–9 months old) were compared to old (36–42 months old) animals during a 6-month dietary intervention by assessing vitamin C transport and redox homoeostasis in the brain. In contrast to our hypothesis, chronic vitamin C deficiency did not affect the measured markers of oxidative stress in the brains of adult and aged animals. However, aged animals generally showed increased lipid oxidation ($p < 0.001$), decreased glutathione ($p < 0.05$), increased *p53* mRNA expression ($p < 0.01$) and somewhat elevated DNA oxidation ($p = 0.08$) compared to adult counterparts irrespective of dietary vitamin C intake. Increased mRNA expression of *sod1* ($p < 0.05$) and *svct2* ($p = 0.05$) was observed in aged animals together with increased superoxide dismutase activity ($p < 0.01$) and cerebrospinal fluid vitamin C status ($p < 0.001$) suggesting a compensatory effort that did not counterbalance the effects of ageing. Essentially, no effects of age were observed in the liver demonstrating the brain's unique susceptibility to redox imbalance. Consistent with previous findings, we show that ageing *per se* constitutes a considerable oxidative insult in the brain. However, our data also suggest that a long-term poor vitamin C status does not accelerate this process.

The overall prevalence of age-associated brain disorders such as Alzheimer's disease, Parkinsonism and dementia is increasing in the Western World as a natural consequence of the improved average lifetime expectancy [1–3]. This has prompted investigations into the underlying mechanisms associated with ageing to become a growing field of interest. One of the areas receiving particular attention and subject to debate is the putative relationship between oxidative stress, the intake of different nutritional factors – such as antioxidants – and the development and prevention of age-related disorders [4]. Increases in cellular reactive oxygen species (ROS) has consistently been reported during ageing [5]. The increase in ROS is reflected by a decreased gene transcription resulting in cellular stress and oxidative DNA damage – as seen in the brain [6] – and oxidative mitochondrial damage followed by mitochondrial dysfunction [7,8]. Furthermore, DNA repair mechanisms are reduced adding further to the harmful consequences of the oxidative damage in the cell [9].

In the brain, vitamin C (ascorbate/ASC) plays a pivotal role in maintaining redox homoeostasis and has additional functions, that is, reducing the risk of neuronal damage because of excess dopamine and glutamate [10]. The brain has one of the highest concentrations of ASC in the body reaching levels 100-fold higher than what is found in plasma [11]. Furthermore, the brain is able to maintain a high level of ASC relative to other tissues during states of deficiency [12], because of the ASC-specific transporter (Sodium dependent Vitamin C co-Transporter 2/*svct2*) found in both the choroid plexus and in neurons [13,14].

In studies of the progression of ageing-associated neurodegenerative disorders, antioxidants have been shown to reduce the effects in both *in vivo* animal models and in human epidemiological surveys [15,16]. Although some studies fail to show a beneficial effect of antioxidant supplementation on brain function in ageing individuals [17,18], a substantial body of evidence points towards supplementation with antioxidants, for example, vitamin C and vitamin E, as having a positive effect on reducing ageing-associated decay of brain function such as a decline in cognitive performance [18–22]. These findings have lead to the common perception that antioxidants are able to prevent or delay ageing-associated neurodegeneration. The guinea pig is a commonly applied and validated *in vivo* model for the investigation of both ASC-associated mechanisms and effects of ageing [23].

In the present study, we wanted to study the effect of ageing as compared to that of a long-term redox imbalance because of vitamin C deficiency. We, therefore, subjected young adult guinea pigs (3 months) and old (36 months) counterparts to a 6-month controlled feeding regimen with

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either low or normal levels of ASC in a 2 × 2 factorial design. Markers of oxidative stress and vitamin C transport were evaluated with the purpose of testing the hypothesis that poor vitamin C status accelerates oxidative stress in the ageing brain.

Materials and Methods

Animals and study design. The study was approved by the Danish Animal Experimentation Inspectorate under the Ministry of Justice. Upon arrival to the animal facility, female Dunkin Hartley guinea pigs (Statens Serum Institut, Allerød, Denmark) each had an IPTT-200 programmable temperature transponder (Plexx, Elst, the Nederlands) implanted subcutaneously in the neck for identification and monitoring of body temperature. Throughout the period, all animals were group-housed in floor bins (min. 2 ft^2/animal) in an enriched environment. Feed and water was provided *ad libitum*. Animals were attended daily by trained staff and observed carefully for any clinical signs of disease. Body weights and temperature monitoring were recorded every 4 weeks. Weight loss is one of the initial, cardinal symptoms of scurvy and would have been expected if the animals in either groups were approaching scorbutic ASC levels. As no difference in body weight was found between animals on the control diet *versus* deficient diet (fig. 1), we are confident that the two dietary groups are comparable in all aspects besides vitamin C concentration. The fact that young animals had an overall lower bodyweight than old animals is more than likely due to young guinea pigs still growing for at least part of the study period and also being more physically active than old animals, therefore, displaying a leaner body composition and not reaching the same total body weight.

At study start, animals were randomized into weight-stratified experimental groups and fed a quality-controlled guinea pig diet (Special Diet Services, SDS, Witham, UK) containing either 325 mg vitamin C/kg feed ('CTRL') or 100 mg vitamin C/kg feed ('DEF') in a 2 × 2 factorial design. Old guinea pigs: Animals were obtained at 12 months of age and group-housed for an additional 24 months prior to entering the study at 36 months of age. Old guinea pigs were kept on the experimental diet for 6 months, Old_{CTRL} (n = 9); Old_{DEF} (n = 16), during which five animals from the Old_{CTRL} and 10 animals from the Old_{DEF} group died, apparently because of old age as confirmed by post-mortem autopsy. Young animals were obtained at the age of 2 months and allowed 1 month of equilibration before entering the study at 3 months of age and assigned to the two different feeding regimens, $Young_{CTRL}$ (n = 5); $Young_{DEF}$ (n = 8). One animal in the $Young_{CTRL}$ group had to be killed during the experimental period because of factors apparently unrelated to the study. Young guinea pigs were kept on the diet for 6 months before termination of the study. Throughout the study period, monthly blood samples (4 × 70 µl) were drawn from *v. saphena lateralis* from all animals. At termination of the study, animals were euthanized by i.p. injection of pentobarbital (55 mg/kg supplemented with 2% Lidocain). Following deep anaesthesia and the concomitant disappearance of voluntary reflexes, thoracotomy was performed and 1000 units of heparin injected intracardially. After 1 min., 5 ml blood was collected by cardiac puncture and processed immediately for analysis (see description below, 'Biochemistry'). Animals were perfused with 100 ml of ice-cold PBS (if not otherwise stated, PBS = Dulbecco's PBS solution, pH: 7.4), prior to the harvesting of liver, cerebrospinal fluid (CSF) and brain, all of which were subsequently frozen to −80°C until further analysis.

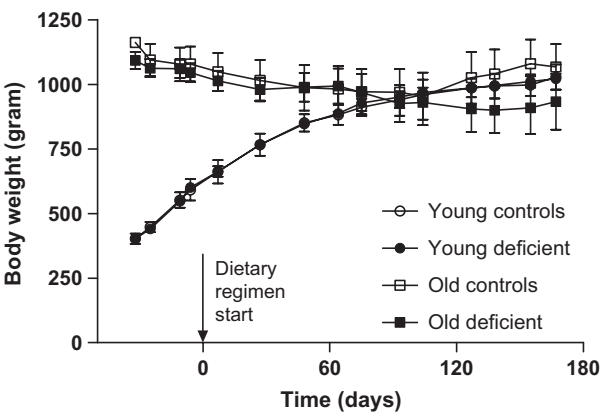

Fig. 1. Body weights of guinea pigs throughout the study.

Biochemical analysis. Blood samples were immediately centrifuged at 2000 × *g* for 5 min. at 4°C. Plasma or CSF: to one aliquot of 25 µl, an equal volume of 10% meta-phosphoric acid (MPA) – containing 2 mM EDTA- was added (Merck, Darmstadt, Germany). Samples were briefly mixed by vortex and stored at −80°C for later analysis of ASC, while remaining plasma samples were stored neat at −80°C. Tissue samples (approximately 0.5 g) were taken from −80°C and homogenized in cold (4°C) PBS and centrifuged (15.000 × *g*, for 1 min. at 4°C). Samples for ASC analysis were stabilized with MPA prior to further analysis. Vitamin C (Fluka, Milwaukee, IL, USA) analysis (equivalent to total ASC) in plasma, CSF and tissue homogenates was performed by HPLC with coulometric detection as described previously [24,25]. Analysis for glutathione (GSH) and superoxide dismutase (SOD) (Sigma, St. Louis, MO, USA) was performed as described by others [26,27]. Malondialdehyde (MDA) was assessed by thiobarbituric acid derivatization followed by specific quantification of genuine MDA-$(thiobarbituric\ acid)_2$ adduct by HPLC with fluorescence detection [28]. Oxidatively modified DNA as measured by 8-oxo-deoxyguanosine content was quantified by HPLC with electrochemical detection as described and expressed per 10^6 unmodified guanosine residues [29].

Gene expression analysis. Samples of brain and liver tissue stored at −80°C were weighed (approximately 20 mg) and homogenized in 1 ml trizol (InVitrogen, Merelbeke, Belgium), followed by centrifuge at 5900 × g (rcf)/3 min., 4°C. Supernatant was transferred to 2 ml tube (Eppendorf), and extraction was initiated by chloroform and isopropanol (Merck) prior to the transfer of supernatant to spin column system (SV Total RNA Isolation System, Promega, Madison, WI, USA) and subsequently proceeding with the Promega spin protocol (step 7 fwd). Extracted RNA was eluted in nuclease-free water and immediately frozen at −80°C. An aliquot from each sample was submitted to triplicate analysis by spectrophotometry (Nanodrop 2000; Thermo Scientific, Wilmington, DE, USA) for the confirmation of RNA purity. For cDNA synthesis, 2 µg of RNA was submitted to RT-PCR [MmLV RT enzyme, 5xMmLV buffer and RNasin (Promega); 10 mM dNTPs and Oligo (dT) primers (60 µg/120 µl) (Fermentas GmbH, St Leon Roth, Germany); Random hexamere primer (2 µg/µl) (GE Healthcare, Uppsala, Sweden)] and performed in duplicates yielding a total volume of 50 µl of cDNA from each sample.

All cDNA samples were submitted to conventional PCR [GoTaq DNA (Promega), 10 mM dNTP, 100 µM primers] with an intron spanning primer set (*β-actin*) and gel electrophoresis (2% agarose gel in TBE-buffer) for the detection of genomic DNA contamination. Only samples which showed no signs of contamination were included in the study. Primers (table 1) were created from guinea pig-specific sequences or from preciously published primer sequences [30]. Conventional PCR and electrophoresis were conducted for all included genes followed by PCR cleanup (PCR Clean Up System; Promega) and subsequent sequencing of PCR product (LGC (AGOWA), Berlin, Germany).

Quantitative PCR (Q-PCR) was performed on a LightCycler LC480 (with SYBR Green I master LC480 and LC480, white 96 well, plates; Roche, Basel, Switzerland). A standard curve was generated from cDNA calibrator dilutions and the acquired efficiency applied in later analysis. Samples were analysed in triplicates, analysis including a negative (nuclease-free water) and calibrator controls. Expression ratios of target genes *svct1, svct2, p53* and *sod1* were obtained by normalization to reference genes *β-actin* and *gapdh*. The total number of samples included for statistical analysis was as follows: $Young_{CTRL}$, n = 4; Old_{CTRL}, n = 4; $Young_{DEF}$, n = 7; Old_{DEF}, n = 6. Despite several attempts to improve extraction, one liver sample from an Old_{CTRL} animal failed to provide mRNA of adequate quality and was thus excluded (Old_{CTRL} Liver, n = 3).

Statistical analysis. The obtained data were analysed by two-way ANOVA using diet and age as factors followed by *post hoc* LSD test in the case of overall effect. Repetitive ASC plasma profiles and weight data were analysed by repeated measures ANOVA. Homogeneity of variances was tested by Levene's test. Unless stated in the text, no interaction was found between diet and age. For data not initially complying with a normal distribution, logarithmic transformation was carried out prior to completing the statistical analysis. A *p*-value of <0.05 was considered statistically significant. All statistical analyses were performed using STATISTICA software (version 9; StatSoft, Tulsa, OK, USA).

Results

Animal physiology.

At the beginning of the study period, animals were assigned to their dietary groups by weight stratification ensuring a high degree of homogeneity between the groups and reducing bias. As part of the general monitoring of the animals, body weight data were obtained routinely throughout the study period (fig. 1). No statistically significant difference was detectable between control and deficient animals ensuring that any recorded divergence between the two dietary groups was not because of overall weight differences. However, young animals did have an overall significantly lower body weight compared to old animals ($p < 0.05$). One $Young_{CTRL}$ animal died during the study and both Old_{CTRL} and Old_{DEF} animals died (5_{CTRL} and 10_{DEF}) because of old age. Survival analysis revealed no statistical evidence to associate the observed mortality rates with the two different dietary groups ($p = 0.28$, *data not shown*).

Table 1.
Overview of genes and primer sequences applied for expression analysis by quantitative PCR.

Gene	Primer sequence	Product size (bp)	NCBI Acc no.
Svct-1	(f): tccgacagattatggcttcc	211	AF410935
	(r): gcacaagcatagtaatcaccga		
Svct-2	(f): gtccatcggtgactacta[1]	114	AF411585
	(r): atgccatcaagaacacagga[1]		
P-53	(f): tgagcagagttaggacagagaagag	315	AJ009673
	(r): ccaagggtcagcaagaggtg		
Sod1	(f): tgtccatgagtttggcgata	193	U39844
	(r): atttgctccggagagtgaga		
β-actin	(f): gtaaggacctctatgccaacaca	346	AF508792
	(r): atgccaatctcatctcgttttct		
Gapdh	(f): gcaccgtcaaggctgagaat	227	DQ403052
	(r): catcacgaacataggggcatc		

All primer sequences are presented in a 5′–3′ direction (f): forward primer, (r): reverse primer. Compliance with NCBI GenBank Accession number has been confirmed for all genes by sequence analysis.
[1]Primers as stated in Clark *et al.* [30]

Biochemistry.

Ascorbate analysis was performed on plasma, CSF and brain and liver samples. Results are presented in table 2. As expected, ASC concentrations were consistently lower in deficient animals compared to controls ($p < 0.001$ in all cases) demonstrating that the deficient animals were indeed exposed to an oxidative challenge. For both the investigated tissue types (brain and liver), no effect of age was observed. However, in the CSF, both diet and age were found to have an influence on the ASC concentration, old animals having a significantly higher ASC concentrations than young animals ($p < 0.001$).

Analyses of lipid and DNA oxidation were performed on brain and liver samples. In the brain, MDA was not significantly different in deficient animals compared to controls, while significantly higher in old animals than young ($p < 0.001$). DNA oxidation measured as 8oxodG is somewhat elevated in the brains of aged animals although not significantly ($p = 0.08$) while GSH was lower ($p < 0.05$). SOD in the brain was not affected by the two different diets, but was significantly increased in old animals ($p < 0.001$). In the liver, MDA concentration was higher in old controls compared to old deficient animals ($p < 0.01$), while this was not the case for young animals. Old animals on deficient diet also had a significantly lower MDA concentration than young animals on equal diet ($p < 0.05$). Liver SOD level was not significantly influenced by the different diets, but in contrast to the brain, it was significantly decreased in old animals compared to young ($p < 0.01$).

Gene expression analysis.

All results are presented as the mean expression ratios between the target gene and two reference genes (*β-actin* and *gapdh*). In the brain, *svct2*, *sod1* and *p53* were analysed; results are presented in fig. 2. Expression of *svct1* in the brain was undetectable (*data not shown*) in accordance with our expectations because *svct2* is the primary vitamin C transporter in the brain. The two feeding regimens did not result in significant alterations in brain mRNA expression ratios of any of the investigated genes. However, both *sod1* and *p53* were significantly increased in old animals compared to young ($p < 0.05$ and $p < 0.01$, respectively) (fig. 2). Expression of *svct2* was higher in old animals compared to young although this difference did not reach statistical significance ($p = 0.05$). In the liver (data not shown), no statistically significant changes in expression ratios could be established for any of the investigated genes in animals of different feeding regimens or of different age groups.

Table 2

Oxidative stress markers in plasma, brain and liver of adult (9 month) and old (42 month) guinea pigs following a 6-month dietary regimen of vitamin C deficiency *versus* control.

	Adult controls (n = 4)	Adult deficient (n = 8)	Old controls (n = 4)	Old deficient (n = 6)	Effect (age)	Effect (deficiency)
Vitamin C						
Plasma (μM)	28 ± 8.7	7.2 ± 5.0	24 ± 6.7	8.2 ± 6.1	ns	***
Cerebrospinal fluid (μM)	99 ± 32	24 ± 19	146 ± 19	35 ± 12	***	***
Brain (μmol/g FW[1])	1013 ± 131	611 ± 185	1023 ± 201	605 ± 47	ns	***
Liver (μmol/g FW)	809 ± 166	308 ± 126	855 ± 196	309 ± 59	ns	***
Malondialdehyde						
Brain (nmol/g FW)	385 ± 50	436 ± 50	511 ± 76	555 ± 63	***	ns
Liver (nmol/g FW)	463 ± 243	528 ± 159	590 ± 254	249 ± 38	ns	ns
Glutathione						
Brain (μmol/g FW)	1054 ± 66	1041 ± 58	870 ± 212	978 ± 95	*	ns
Liver (μmol/g FW)	3242 ± 358	2821 ± 333	2353 ± 417	2973 ± 393	ns	ns
8-oxodG						
Brain ($1/10^6$dG)	2.8 ± 1.1	2.7 ± 2.0	4.7 ± 1.2	3.4 ± 1.1	ns	ns
Liver ($1/10^6$dG)	5.4 ± 1.0	5.1 ± 2.5	4.7 ± 1.6	7.2 ± 2.6	ns	ns
Superoxide dismutase						
Brain (μg/g FW)	112 ± 24	115 ± 19	145 ± 17	139 ± 14	**	ns
Liver (μg/g FW)	954 ± 159	1106 ± 122	827 ± 102	851 ± 197	**	ns

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by two-way ANOVA using age and diet as factors.
[1]FW: Fresh Weight.

Discussion

The brain has been repeatedly investigated to establish a causal cascade leading to deterioration of brain function in ageing individuals. In the present study, we wanted to compare the effect of ageing itself to that of a marked long-term redox challenge: vitamin C deficiency. ASC is important in several brain processes: as a scavenger of cellular free radicals, as an enzymatic co-factor in the synthesis of neuronal transmitter substances [31] and in the prevention of glutamate excitotoxicity through the glutamate-ASC exchange system [32]. We have recently hypothesized that cellular damage and apoptosis may occur through all of the above pathways, rendering particularly the developing brain susceptible to deficiencies in vitamin C [10]. The apparently increased susceptibility of the brain compared to other tissues in the organism is supported by the brain's ability to selectively retain ASC by the SVCT-2 acting as a high-affinity-low capacity transporter system and enabling the CNS to maintain a high ASC concentration relative to plasma and other tissues during states of deprivation [12,33].

In accordance with our expectations, plasma and brain concentrations of ASC reflected the applied diets. However, in contrast to our hypothesis that a prolonged state of low ASC concentrations would lead to an increased redox imbalance further enhanced by ageing, vitamin C deficiency itself did not significantly affect any of the measured markers of oxidative stress in the brain (table 2). In the CSF, concentrations varied because of both diet and age, old animals having a relatively higher ASC content than young. This has also been reported in aged human dementia patients indicating an increased requirement of vitamin C in the brain as ageing progresses [34]. MDA concentrations in the brain were apparently slightly elevated in animals on vitamin C deficient diet compared to controls, but this increase did not reach statistical significance. In line with this, no diet induced differences were seen for SOD or mRNA expression levels of target genes. In contrast, we previously found a massive 2.5-fold increase in brain MDA in severely deficient neonatal guinea pigs *versus* controls linking ASC deficiency to oxidative damage in the brain [12]. This condition is also known to induce cellular DNA modifications in ageing [35]. Our findings suggest that the ageing brain is well protected against a redox-mediated damaging effect of vitamin C deficiency.

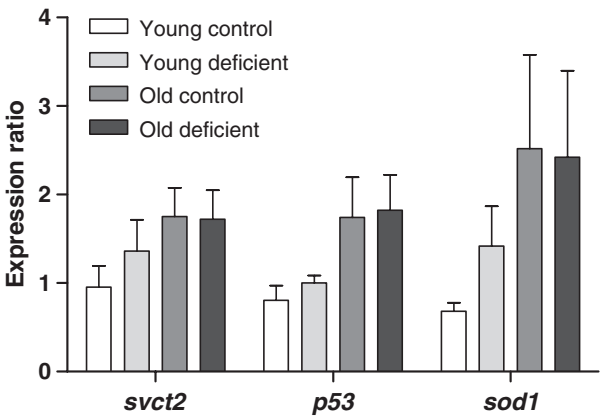

Fig. 2. Quantitative-PCR analysis of mRNA expression of target genes in brain tissue. Expression ratios are presented as a mean of the target genes' normalized ratio of reference genes *β-actin* and *gapdh*. No significant differences ($p > 0.05$) in mRNA expression because of the two feeding regimens were found; however, both *sod1* and *p53* were increased in old animals. *Svct2* expression was not significantly increased in old animals compared to young ($p = 0.05$). *$p < 0.05$; **$p < 0.001$.

A reason for the lack of effect in the present study may be the chosen dietary levels of vitamin C. Both diets resulted in

relatively low ASC plasma levels from a human perspective where the deficient diet resulted in 'severe vitamin C deficiency' while the controls were just above the human threshold for hypovitaminosis C (23 μM). This threshold has been set empirically for human beings and does not necessarily represent a relevant threshold in guinea pigs. However, the two diets did not result in plasma levels of ASC that were very far apart which may explain why there was no significant effect of diet in several of the examined parameters. If the ASC plasma level had been higher in the control animals, that is, about 70-80 μM that constitutes a level of saturation in human beings, it is possible that a substantial divergence to the deficient guinea pigs would have been observed. However, the diets did result in an approximate 75% decrease in CSF ASC and a 40% lower brain ASC concentration. It is, therefore, likely that the brain's buffer capacity with regard to vitamin C is greater in adult and aged animals than during development. However, taken together with the recorded reductions in ASC of brain tissue homogenate and CSF in deficient animals compared to controls, it appears that only more substantial reductions in plasma ASC will have a pronounced effect on the redox homoeostasis in the brain.

In contrast to vitamin C deficiency, ageing *per se* produced a far more pronounced effect on redox homoeostasis in the brain. Aged animals showed increased lipid oxidation ($p < 0.001$), decreased GSH ($p < 0.05$), increased *p53* expression ($p < 0.01$) and somewhat elevated DNA oxidation ($p = 0.08$) in the brain compared to their adult counterparts. Meanwhile, essentially no effects of age were observed in the liver thereby demonstrating the brains' unique susceptibility to redox imbalance. In agreement, we have previously found the liver to be more resistant to oxidative damage than the brain [12]. The progression of neuronal death in the ageing brain and in Alzheimers Disease has been linked to increased oxidative stress and changes in *p53*, an upstream effector of the apoptotic pathway [36–38]. An increase in cellular *p53* has been suggested to induce excitotoxic neuronal death through both apoptosis and autophagia [39]. Thus, the observed increased expression level of *p53* in old animals in the present study supports the biochemical findings of redox imbalance in the brain and is indicative of concurrent neuronal injury.

Increased mRNA expression of genes encoding for *Sod1* ($p < 0.05$) and the vitamin C transporter *svct2* ($p = 0.05$) was observed in the brains of aged animals showing that the observed changes in cellular mechanisms in the old animals take place even at a transcriptional level. An up-regulation of *svct2* has been demonstrated following cerebral ischaemia in rats, suggesting vitamin C as a neuroprotective agent and increased transport as an immediate response to neuronal injury possibly through excitotoxic ROS generation and mitochondrial hyperoxidation [40–42]. Likewise, the corresponding increases in SOD activity ($p < 0.01$) and CSF vitamin C status ($p < 0.001$) found in our study support the above findings and possibly represents a joint compensatory response that, however, did not manage to counterbalance the effects of ageing.

In conclusion, our findings support the notion that ageing *per se* constitutes a considerable oxidative insult particularly in the brain that may be an underlying mechanism in cognitive decline. In contrast to our expectations, the reported changes were independent of vitamin C levels, thus showing no significantly beneficial effects of vitamin C within the chosen dietary regimens on brain redox homoeostasis during ageing. Importantly, the present study did not assess cognitive or neuronal function, and therefore, it remains to be established if poor vitamin C status could translate into clinical deficits associated with ageing.

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