Vitamin C deficiency in early postnatal life impairs spatial memory and reduces the number of hippocampal neurons in guinea pigs

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ABSTRACT

Background: The neonatal brain is particularly vulnerable to imbalances in redox homeostasis because of rapid growth and immature antioxidant systems. Vitamin C has been shown to have a key function in the brain, and during states of deficiency it is able to retain higher concentrations of vitamin C than other organs. However, because neurons maintain one of the highest intracellular concentrations of vitamin C in the organism, the brain may still be more sensitive to deficiency despite these preventive measures.

Objective: The objective was to study the potential link between chronic vitamin C deficiency and neuronal damage in newborn guinea pigs.

Design: Thirty 6- to 7-d-old guinea pigs were randomly assigned to 2 groups to receive either a vitamin C–sufficient diet or the same diet containing a low concentration of vitamin C (but adequate to prevent scurvy) for 2 mo. Spatial memory was assessed by the Morris Water Maze, and hippocampal neuron numbers were quantified by stereologic techniques.

Results: The results showed a reduction in spatial memory ($P < 0.05$) and an increased time to first platform hit ($P < 0.05$) in deficient animals compared with controls. The deficient animals had a lower total number of neurons in hippocampal subdivisions (dentate gyrus, cornu ammonis 1, and cornu ammonis 2–3) than did the normal controls ($P < 0.05$).

Conclusions: Our data show that vitamin C deficiency in early postnatal life results in impaired neuronal development and a functional decrease in spatial memory in guinea pigs. We speculate that this unrecognized effect of vitamin C deficiency may have clinical implications for high-risk individuals, such as in children born from vitamin C–deficient mothers. Am J Clin Nutr 2009;90:540–6.

INTRODUCTION

Vitamin C deficiency—defined as a plasma concentration $< 23 \mu$mol/L—is surprisingly common within the human population of the Western world (1–4), although cases of clinical scurvy are rarely reported. Vitamin C–deficient subpopulations include pregnant women sharing blood vitamin C with their fetus, mothers, who convey their vitamin C deficiency to newborns during breastfeeding (5), and a recent Mexican study found severe vitamin C deficiency in $\approx 30\%$ of young children (age 0–2 y) (6). Although not specifically related to vitamin C, infants exposed to intrauterine growth restriction have increased neuronal degeneration and learning disabilities (7, 8), and other studies have shown that supplementation with antioxidants such as vitamin C may improve survival in preterm babies (9, 10).

In the developing brain, chronic malnutrition is a well-established contributor to deviation in neuronal function, including the development of the hippocampus (11, 12) and cognitive impairment in both humans and in vivo animal models (13–16). In guinea pigs, fetal malnutrition results in reduced numbers of hippocampal and cerebellar neurones (11), linking malnutrition to neuronal damage in the developing brain as well as the possible involvement of micronutrient deficiencies. Despite these reports, no detailed studies exist on the possible consequences of chronic, nonscorbutic vitamin C deficiency and its potential effect on brain development. However, studies by us have shown that young guinea pigs are more prone to oxidative stress than are mature animals (17) and that the developing brain of neonatal guinea pigs is particularly susceptible to vitamin C deficiency because of rapid growth and an immature antioxidant defense system (18). Thus, we recently found that only the brain had increased oxidative damage during severe vitamin C deficiency, despite much more profound depletion in other tissues (18). Embryonic sodium-dependent vitamin C cotransporter 2 (SVCT2) knockout cells have a reduced growth rate and an increased susceptibility to excitotoxicity and oxidative damage in vitro, linking intracellular ascorbate homeostasis to neuronal development and function (19). Indeed, mice devoid of a functional SVCT2 transporter do not survive beyond infancy (20) and display pathologic signs resembling findings in premature human infants, the latter being associated with decreased learning competency (21).

On the basis of the strict control of vitamin C homeostasis, the ability of the brain to selectively retain vitamin C during deficiency and the increased sensitivity of the neonatal brain to oxidative stress, we hypothesized that adequate concentrations of vitamin C are important for normal brain development (4).
Because of the lack of L-gulono-γ-lactone oxidase, guinea pigs—like humans—are dependent on an exogenous vitamin C source (22) and are a validated animal model of vitamin C deprivation. In this study, we assess the consequences of nonscorbutic, chronic vitamin C deficiency in early postnatal life in guinea pigs. The functional outcome was evaluated by testing memory and the ability to navigate spatially in the Morris Water Maze (MWM), whereas neuronal development of the hippocampal regions was quantified by stereologic techniques.

**MATERIALS AND METHODS**

**Animal feeding and housing conditions**

The study was approved by the Danish Animal Experimentation Inspectorate under the Ministry of Justice. On arrival, a total of 30 female, 6–7-d-old Dunkin Hartley guinea pigs (HA-SIFIE150200; Charles River Laboratories, Kisslegg, Germany) were weighed and tagged with a 12-mm microchip subcutaneously in the neck for identification (Pet-Id; Danworth Farm, West Sussex, United Kingdom). Animals were randomly assigned into 2 weight-stratified experimental groups (initial weight: 194.8 ± 14.9 and 193.1 ± 17.8 g for controls and deficient animals, respectively) that received special quality-controlled diets either adequate in vitamin C (923 mg/kg by analysis; product code: 829427-B#32786; control group, n = 15) or low in vitamin C (100 mg/kg by analysis; product code: 829415-B#32785; deficient group, n = 15). The diets were produced to meet the nutritional requirements of guinea pigs and were identical in all aspects except vitamin C content. They were based on a blend of raw materials in standard guinea pig feed compressed into pellets of 4 mm in diameter (Special Diets Services; Dietex International Ltd, Witham, Essex, United Kingdom). The deficient diet did not result in signs of scurvy and gave rise to plasma concentrations of ~8.5 μmol/L (Table 1), resembling those observed in humans with chronic severe vitamin C deficiency (defined as a plasma concentration <11 μmol/L). Groups were allowed ad libitum feeding, dried hay (containing negligible amounts of vitamin C by analysis), and drinking water and were kept under identical housing conditions with a 12-h light cycle. The animals were handled systematically once a day to reduce handling stress and were weighed twice a week. Blood samples (300 μL) were collected every 4 wk without the use of an anesthetic from a saphenous vein at its superficial course in the tibia for verification of vitamin C status (data not shown). During the first month, 3 deficient animals were killed because of teeth problems. Abnormal incisor growth was previously observed as a result of vitamin C deficiency (23). However, in the present study, the problems were not associated with the incisors but rather with the molars, which resulted in food accumulation in the mouth, immobilization of the tongue, and starvation. In a recent study by us, a uniform distribution of the same molar problems was observed regardless of vitamin C status (n = 21 in each group), and most of the animals had to be killed (Tveden-Nyborg et al, unpublished observations, 2008). A change in the animal supplier largely eliminated the problem, which appears to be of genetic origin. On the basis of our investigation of the problem, it was concluded that the teeth problems in the present study were most likely not attributed to the dietary regimen.

**Morris Water Maze**

The overall procedures applied in the MWM were described in detail by others (24–26). In brief, we used a 1.5-m-diameter dark pool filled with 24°C water to a depth of 42 cm, which covered the platform (30 cm in diameter). The hidden platform was placed away from the wall of the pool and in the center of 1 of the 4 quadrants. Distal cues were mounted on the surrounding outside walls. A total of 27 animals (controls, n = 15; deficient, n = 12), aged 52–53 d were trained in the MWM during the 5-d acquisition phase (hidden platform in quadrant Q2), followed by a 4-d resting phase. On day 5, animals were subjected to the retention test without the platform present in the pool, followed by a task familiarity test. All MWM tests were performed with animals in a randomized order and at randomized starting points (except the retention test in which the starting point was kept uniformly in quadrant Q1) (24). On completion of each test, the animals were dried and placed beneath a heating lamp. All swimming sessions were recorded with a digital camera suspended above the MWM pool (Camcolmha3; Velleman, Gaverer, Belgium), which was connected to a data acquisition computer that stored the sessions in mpg4-format. The various MWM variables were subsequently obtained by analyzing the sessions with Ethovision 3.1 (Noldus Information Technology, Wageningen, Netherlands).

**Euthanasia and tissue collection**

At 60–61 d of age, the animals were anesthetized with 0.175 mL/100 g body weight of supplemented zoletil suspension (0.465 mg/mL Zoletil 50 vet; Virbac SA, Carros Cedex, France), 2 mg/mL Xylazin (Narcyxyl; Intervet Int, Boxmeer, Holland), and 1 mg/mL butorphanol tartrate (Torbugesic; ScanVet, Fredensborg, Denmark) in isotonic sodium chloride. To ensure adequate anesthetic depth, the animals were supplemented with brief carbon dioxide inhalation until the disappearance of voluntary (interdigital and palpebral) reflexes. Immediately thereafter, thoracotomy and subsequent intracardial blood sampling...
was performed by using a 5-mL syringe and an 18-G needle previously flushed with 15% tripotassium-EDTA. The animals were killed by exsanguination. The final body weights were 528 ± 39 and 479 ± 35 g for the control and deficient animals, respectively (P < 0.05 by repeated measures ANOVA of all weight data). Blood samples were immediately centrifuged and stabilized after sampling. Organ and tissue samples were removed and placed in ice-cold phosphate-buffered saline before being frozen on dry ice and subsequently at −80°C. The brain was excised from the cranium case and was divided into left and right hemispheres (sagittal section through the cerebral longitudinal fissure) and randomly assigned to either fixation/stereology or biochemical analyses.

Biochemical analysis

Ascorbate and dehydroascorbic acid concentrations in meta-phosphoric acid–stabilized plasma and brain tissue were analyzed by HPLC with coulometric detection as described previously (27, 28). Analyses of brain glutathione and superoxide dismutase were performed according to Marklund and Marklund (29) and Hissin and Hilf (30), respectively. Malondialdehyde was used as an index of lipid oxidation and was assessed by thiobarbituric acid (thiobarbituric acid)2 adduct by HPLC with derivatization, followed by specific quantification of the genuine index of lipid oxidation and was assessed by thiobarbituric acid and Hilf (30), respectively. Malondialdehyde was used as an index of lipid oxidation and was assessed by thiobarbituric acid derivatization, followed by specific quantification of the genuine index of lipid oxidation and was assessed by thiobarbituric acid (thiobarbituric acid)2 adduct by HPLC with fluorescence detection as described previously (31).

Stereologic analysis

Randomized (left compared with right) hemispheres from 9 deficient and 13 control animals were fixed in 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) at 4°C for 4 d before 2 h of preinfiltration (hydroxymethyl acrylate, Technovit 7100; AX-Laboratory, Vedbaek, Denmark) and infiltration at 4°C for 10 d (1 g/100 mL glucomethyl acrylate, Technovit 7100). The hemisphere was divided horizontally and embedded in one block in Technovit 7100. Three hemispheres were eliminated from the examination for technical reasons, which left 8 deficient and 11 control brains for stereologic quantification. The block was sectioned exhaustively into 40-μm serial sections (Finesse microtome; Thermo, Shandon, United Kingdom), and every ninth section was sampled systematically, placed on coated glass (Superfrost +), left at 60°C overnight, and stained in a modified Giemsa stain (32). Overall hippocampal morphology was evaluated by light microscopy and found to be in coherence with structural hippocampal subdivisions as reported previously (33–35). Stereologic quantification was performed by light microscopy (BX51 Olympus and CAST-grid software; Olympus, Ballerup, Denmark) by using the optical fractionator technique (32). Neurones were counted in optical dissectors with an ×100 oil objective using the nucleolus as the counting unit. In cases of multiple nucleoli, the largest and most prominent was chosen; if equal in size, the nucleolus to the right was chosen. The optical dissectors were positioned systematically, randomly in a raster pattern on the section by stepping motors that move the section in fixed steps of length, dx and dy in the x and y axis, respectively. The optical dissectors thus constituted a fixed fraction of the sections, namely the volume of the dissector, ie, the area of the unbiased counting frame, a(frame), times the height of the dissector, h(dis), divided by the section volume defined by dx times dy times section thickness, t (section).

An estimate of the total number of neurons (N) was obtained by multiplying the number of neurons counted (Q−), in the fixed fraction by the inverse sampling fraction:

$$N = \text{ssf'}[dx'dy't(\text{section})/a(\text{frame})'h(dis)]'Q^- \quad (1)$$

where ssf is the section sampling fraction, ie, 1/9. All 3 subregions were counted by using an unbiased counting frame with an area of 29.6 μm2. The step lengths in the x and y directions were 160 μm for the dentate gyrus, 90 μm for cornu ammonis 1, and 100 μm for cornu ammonis 2–3. The section thickness was measured systematically over the sections with the microcator for every 2000 times 2000 μm.

Statistics

Statistical calculations were carried out by using StatSoft Statistica (version 7; Tulsa, OK). Animal weights and data from the MWM tests were analyzed by repeated-measures ANOVA (acquisition phase). Data from the MWM retention tests were analyzed by one-factor ANOVA or by the Mann-Whitney U test for data that was not normally distributed. Allocation to swimming patterns was tested by using a chi-square test. Stereologic data for each hippocampal region were compared by using the appropriate unpaired t test after testing the group variances with the F test. The biochemical data were analyzed by one-factor ANOVA, and the data are expressed as means ± SD. A P value <0.05 was considered statistically significant.

RESULTS

Biochemical analysis

The results of the biochemical analyses are reported in Table 1. The biochemical analyses verified the vitamin C status of the animals. Plasma ascorbate concentrations were in line with published data, which confirmed that the ascorbate feeding regimen results in concentrations comparable with those of healthy humans (36) and those of nonscorbutic, hypovitaminosis C (37), respectively. Dehydroascorbic acid was higher in the plasma (P < 0.001) and brain tissue (P < 0.05) of deficient animals than in the controls, which indicated an inadequate recycling capacity during vitamin C deficiency. Ascorbate concentrations in the brain decreased by >50% as a result of the dietary regimen (P < 0.001). No change in glutathione status, superoxide dismutase, or malondialdehyde was observed as a result of the imposed vitamin C deficiency (Table 1).

Morris Water Maze

During the 5-d acquisition phase, we found no significant difference between the 2 groups (data not shown). Likewise, the task familiarity test showed no difference between the 2 groups in the ability to learn a new location of the platform (data not shown). However, significant differences were found with the retention test. Swim paths were analyzed for 4 performance measures: total time spent in Q2, crossings of the original position of the platform (PP) in Q2, time before PP was initially reached, and overall average distance to PP during trial (Figure 1).
Further blinded analysis of swim patterns into spatially persistent, concentric circles, or random according to Dalm et al (38) showed a clear effect of vitamin C deficiency \( (P < 0.001) \) (Figure 2). Elimination of animals that swim in concentric circles from the statistical analyses above to minimize the risk of false-spacial swim patterns, as suggested by Dalm et al (37), all performance measures from the Morris Water Maze retention test were significantly different between groups (data not shown).

**Hippocampal neuron number**

In all 3 subdivisions of the hippocampus, there was a significantly \( (P < 0.05) \) lower number of neurons in the deficient than in the controls animals (Table 2). Testing for correlation between body weight and total number of neurons within the 3 hippocampal subdivisions showed no association \( (P = 0.5) \).

**DISCUSSION**

The present study examined the hypothesis that vitamin C deficiency in early postnatal life compromises normal brain development in guinea pigs. Active SVCT2 (39) is responsible for ascorbate uptake in the cerebrospinal fluid, which sustains an ascorbate concentration in the cerebrospinal fluid of \( \approx10 \)-fold that of plasma (40, 41). Ascorbate enters the extracellular fluid by diffusion from the cerebrospinal fluid or through capillaries in the blood-brain barrier, possibly through facilitated GLUT transport (42). Relatively high concentrations of vitamin C in the brain during reduced plasma and tissue ascorbate concentrations indicate that the brain is favored during deficiency, which indicates an essential role of vitamin C in the brain (18, 43).

Vitamin C-deficient knockout mice exhibit sensorimotor disabilities associating neuronal dysfunction with increased oxidative stress (44). Neuronal quantities of vitamin C exceed those of all other tissues, reaching concentrations of 10 mmol/L (45). Cellular uptake in the brain is achieved by simple diffusion, facilitated by glucose coupled transport of dehydroascorbic acid by the GLUT1 and GLUT3 transporters (46, 47) and by the high-affinity SVCT2, which is responsible for the vast majority of neuronal vitamin C uptake and is widely distributed in the brain and choroid plexus (39, 48). In neurons, vitamin C acts to maintain redox homeostasis but is also a key component in the glutamate-ascorbate heteroexchange (49) and in the conversion of dopamine to norepinephrine by dopamine-\( \beta \)-hydroxylase in catecholamine-synthesizing neurons (50). We recently hypothesized that deficiencies of vitamin C may impede neuronal functions by compromising cellular viability, which possibly leads to apoptosis through all 3 pathways (4).

The biochemical analyses showed a pattern of altered redox homeostasis in the brain as indicated by a lower ascorbate and higher oxidation ratio (% dehydroascorbic acid) in deficient than in control animals (Table 1). However, in agreement with our previous studies, no other changes in antioxidants were observed in the brain (18). During severe vitamin C deficiency, we have reported increased oxidative damage in the brains of neonatal guinea pigs based on malondialdehyde concentrations (18). In the present study, no difference in brain malondialdehyde was observed between groups, which suggests that the vitamin C deficiency imposed on these animals was not sufficiently severe to induce oxidative damage (Table 1). Thus, our data suggest that only vitamin C homeostasis was significantly affected by the dietary regimen.

In agreement with our hypothesis (4), vitamin C deficiency impaired normal development in guinea pigs as tested by spatial memory in the MWM. The MWM data yielded statistically significant differences in the retention test (Figure 1), which indicated a reduced ability in deficient animals to apply spatial memory as a means to relocate the platform area. When evaluating performance in the MWM trial in a blinded fashion, swim patterns were allocated as spatial, concentric, and random (38). Only animals complying with a spatial swim pattern were accepted to be applying specific spatial memory in locating the platform area. A concentric strategy (ie, swimming predominantly at a fixed distance to the edge of the pool wall) does...
not uniformly use spatial memory, but animals can falsly be ranked as having a spatial swim pattern. Animals used in a random swim pattern will not apply their spatial memory in navigating in the MWM and show no preference for any of the MWM quadrants. In this study, deficient animals showed a significantly reduced ability to use spatial navigation (Figure 2). Exclusion of animals with a concentric swim pattern further emphasized the difference between groups (Figure 1). Because animals in both groups had equivalent swim times during the MWM acquisition phase and the task familiarity test (data not shown), we concluded that the groups were equally competent in physical ability and motor function to perform the trial, despite a slightly albeit significantly lower weight in the deficient animals. We therefore interpreted the recorded differences in the MWM retention test to be due to an impairment in spatial memory in infant guinea pigs submitted to chronic vitamin C deficiency.

Acquisition and retention of spatial memory is particularly dependent on a functional hippocampus (51, 52); impairment of the hippocampal and extrahippocampal areas of the brain responsible for spatial navigation and path integration is considered to be directly associated with impairment in the MWM (53, 54). In developing guinea pigs, hippocampal dysfunction, as measured by a reduction in cornu ammonis 1 (CA1) neurons, is recognized as a contributor to cognitive and behavioral deviations (26). The clear difference between groups observed in the MWM test prompted us to look for changes in the hippocampal area of the brain. To investigate whether our findings from the MWM test prompted us to look for changes in the hippocampal area of the brain, we stereologically quantified neurons in the hippocampus. To further investigate hippocampal subdivisions in deficient animals than in controls (Table 2). Axons in the CA3 area have been suggested to be directly involved in the recollection of spatial memory, and reductions lead to impaired performance in the MWM retention test (55). Furthermore, we found a correlation between hippocampal neuron numbers in the various subdivisions, ie, animals with high numbers in one subdivision also had high numbers in the 2 other investigated subdivisions (data not shown).

Altricial mammals (eg, humans, rats, and rabbits) are immature at birth and are subjected to extensive postnatal development. Guinea pig, however, are precocial mammals, ie, animals that are able to move freely immediately after birth and undergo early maturation of the brain, reaching a developmental stage at birth comparable with 21-d-old rats and rabbits (56, 57). Immunohistochemical labeling of the hippocampus has established the newborn guinea pig to be, in some aspects, equivalent to adults, which suggests a well-developed spatial memory even at a very young age (56). Assessment of the postnatal proliferation of granule cells in the dentate gyrus has established that brain development in guinea pigs is more comparable with that in primates than in mice and rats (58).

Earlier reports have shown that hippocampal neurones undergo continuous proliferation (59) and that exercise and learning in an enriched environment (rats) can induce this proliferation (60–62). Our stereologic findings may consequently be the result of a decreased vitamin C–dependent proliferation rate; thus, the differences could be even more pronounced in animals not submitted to training in a MWM system. However, it should be noted that the present study could not distinguish between whether vitamin C deficiency mediates increased cell death or decreased proliferation. Despite this problem, data from mice indicate that regenerative capacity is greater in the juvenile than in the immature brain (63).

In rat models of preterm infant brain hypoxia, which is known to result in cognitive and learning disabilities in children, a reduction in neurons of up to 30% and associated decreased performance in the MWM has been reported (64, 65). In animal models of neuronal impairment due to prenatal ethanol exposure, decreases in the hippocampal neuron count of as much as 50% in the rat and of as much as 30% in the CA1 area of guinea pigs has been reported (66, 67), which has been proposed to lead to serious brain dysfunction in children with fetal alcohol syndrome. Our data indicated a reduction in hippocampal neurons of ≈30% in all 3 investigated subdivisions. Because our findings are quantitatively comparable with those of neuronal impairment in other animal models, which is associated with learning disabilities in human infants and children, our data suggest that vitamin C deficiency is likely to be of particular importance in early-life nutrition.

In conclusion, our results indicate reduced spatial memory and impaired hippocampal development in guinea pigs subjected to chronic vitamin C deficiency, which represents a previously unrecognized clinical outcome of hypovitaminosis C. Although a direct extrapolation of this new phenomenon to humans is not currently possible, we found that the relatively high prevalence of vitamin C deficiency in humans, including infants and toddlers (5, 6), warrants future clinical studies to clarify whether a similar link to brain development exists in humans. We speculate that the lack of vitamin C supplementation in high-risk individuals, such as pregnant women and newborns with poor vitamin C status, could be detrimental to normal brain development and lead to neurologic disabilities later in life.

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