NUTRITION RESEARCH 36 (2016) 696–702

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Communication

Maternal vitamin C deficiency does not reduce hippocampal volume and ß-tubulin III intensity in prenatal Guinea pigs

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ARTICLE INFO

Article history:
Received 13 October 2015
Revised 3 March 2016
Accepted 4 March 2016

Keywords:
Hippocampus
Fetal development
Ascorbic acid
Deficiency
Cavia porcellus

ABSTRACT

Marginal vitamin C (vitC) deficiency affects 5% to 10% of adults including subpopulations such as pregnant women and newborns. Animal studies link vitC deficiency to deleterious effects on the developing brain, but exactly how the brain adapts to vitC deficiency and the mechanisms behind the observed deficits remain largely unknown. We hypothesized that vitC deficiency *in utero* may lead to a decreased neuronal maturation and increased cellular death giving rise to alterations of the hippocampal morphology in a guinea pig model. Brains from prenatal guinea pig pups (n = 9-10 in each group) subjected to either a sufficient (918 mg vitC/kg feed) or deficient (100 mg vitC/kg feed) maternal dietary regimen were assessed with regards to hippocampal volume and β-tubulin isotype III staining intensity at 2 gestational time points (45 and 56). We found a distinct differential regional growth pattern of the hippocampus with a clear effect of gestational age, whereas vitC status did not affect either investigated parameters. Within hippocampal subdivisions, the overall expansion of the hippocampus from gestational day 45 to 56 was found to reside in the dentate gyrus. In conclusion, the present study found that hippocampal volume and β-tubulin isotype III intensity in the prenatal guinea pig were influenced by gestational day but not by maternal vitC intake.

1. Introduction

Vitamin C (vitC) deficiency, defined as a plasma concentration below 23 μmol/L [1], affects as much as 5% to 10% of adults in the Western world [2–5], with certain subgroups such as smokers, people with low socio-economic status, pregnant women and their newborns displaying an even higher prevalence [6–9]. Though potentially affecting millions of people globally, aside from scurvy (the very rarely occurring result of severe and prolonged vitC depletion), no clinical effect of vitC deficiency is presently recognized. However, consistent findings from experimental animal models of both

Abbreviations: DHA, Dehydroascorbate; GD, Gestational day; GLUT, Glucose transporter; TB3, β-Tubulin isotype III; vitC, Vitamin C; SVCT2, Sodium dependent vitamin C co-transporter.

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http://dx.doi.org/10.1016/j.nutres.2016.03.004
0271-5317/

vitC depletion and deficiency have suggested vitC as playing a crucial role in the brain, particularly during development [10–14].

Like humans, guinea pigs rely on a dietary vitC source [15]. As a precocial species, the peak of the brain growth-spurt occurs around the last third of gestation, sharing a higher resemblance to humans compared to, eg, mice and rats [16,17]. However, details of prenatal brain development of the guinea pig are scarce. We have previously shown persistently reduced brain weight, redox imbalance and irreversible reductions of hippocampal volume in guinea pig pups subjected to vitC deficiency (non-scorbutic but well below 23 μmol/L) during gestation [14,18,19]. A link between vitC deficiency in early life and reduced memory functions has also been suggested [13]. Studies in mouse models have shown vitC to be crucial to perinatal survival and for deficiency to impair neurotransmitter synthesis and increase oxidative stress [10,20–22]. These proposed links between vitC deficiency and histological, molecular, and functional alterations in the central nervous system of animal models raise the concern for the pre and postnatal cognitive development of children—potentially at risk of vitC deficiency [6,7].

In comparison with most other organs, the brain has an extraordinary ability to maintain high vitC levels despite a low dietary intake [23,24]. Brain levels are primarily controlled by active transport of ascorbate (the reduced form of vitC) through the membrane bound sodium dependent vitC co-transporter (SVCT2) and by efficient intracellular recycling converting dehydroascorbate (DHA)—the oxidized form of vitC—to ascorbate [21,25]. In turn, DHA can be transported across cell membranes facilitated by glucose transporters 1 and 3 (GLUT1 and 3) [26–30]. Neurons express SVCT2 and GLUT3, the latter possibly allowing for the export of excess DHA. However, besides from specific, regional differences, most astrocytes do not express SVCT2 but take up DHA through GLUT1 [29,31–33], subsequently recycling DHA to ascorbate, which may enter the extracellular space, allowing for SVCT2 mediated uptake by adjacent neurons [28,34]. Thus, a closely controlled interplay between the cell types of the central nervous system appears to be involved in the complex regulation of brain vitC homeostasis in vivo. However, the exact transport mechanisms and in which way this might be affected by vitC deficiency remain undisclosed [12,26,34–37]. Apart from maintaining brain redox balance, vitC is associated with specific functions such as preventing glutamate excitotoxicity, biotransformation and synthesis of catecholamine neurotransmitters [38–40]. Furthermore, vitC has been associated with the expansion of dendritic branching and subsequent neurite formation in vitro, suggesting vitC as a key player in normal neuronal development and signal transduction [39,41,42]. Thus, vitC deficiency may negatively impact several important cellular processes in the developing brain, potentially leading to irreversible consequences.

Based on previous findings in our guinea pig model, we hypothesized that chronic, non-scorbutic, maternal vitC deficiency would compromise fetal hippocampal development leading to reduced volume, and deviations in neuronal maturation and mossy fiber formation at late gestational time points—gestational day (GD) 45 and 56 [14,18,19]. Hippocampal volume was estimated using the Cavalieri principle on serial sections from fetal brains excised at the two gestational time points from dams subjected to either deficient (100 mg/kg) or control (918 mg/kg) levels of vitC in the diet [18,43]. The staining intensity of β-tubulin isotype III (TB3) in *stratum lucidum* of the mossy fiber pathway from the dentate gyrus was assessed as a potential indicator of neuronal immaturity, since TB3 is expressed by post-mitotic neurons close to their final destination and the expression decreases with increasing maturation of the central nervous system [44,45].

2. Methods and materials

2.1. Animals

The experimental set-up for the in vivo study adheres to the guidelines of EU Directive 2010/63/EU and was approved by Danish Animal Experimentation Inspectorate as previously described [19]. In short, Dunkin Hartley guinea pig dams (Charles River Laboratories, Kissleg, Germany) at GD 6 to 10 were randomized into 4 groups (n = 5) stratified for weight and GD and allocated to receive either a control (918 mg vitC/kg feed—as determined by postproduction analysis) or deficient (100 mg vitC/kg feed by titration of 0 mg vitC/kg feed with 300 mg vitC/kg feed) diet (SNIFF Spezialdiäten GmbH, Soest, Germany). We have shown these dietary levels to ensure high vitC levels (control diet) and induce a non-scorbutic vitC deficiency (deficient diet) in guinea pigs [13,14,46,47]. The compositions of all 3 diets are shown in Table 1. Nonconceivers (n = 3) were excluded, leaving 4 animals in 3 of the groups. Cesarean section at either GD 45 or 56 was performed as previously described [19]. Weight and sex were recorded for all viable pups and blood was collected by cardiac puncture, after which the pups were immediately euthanized by decapitation [18,19]. The brains were removed, weighed, and fixated in paraformaldehyde (4% for 24 hours, then 1%) before embedding in paraffin. A total of 9 animals were included in each of the GD 45 deficient and GD 56 control group, and 10 animals were included in each of the GD 45 control and GD 56 deficient group.

2.2. Tissue analysis

Paraffin-embedded brains (n = 9 in GD 45 deficient and GD 56 control groups and n = 10 in GD 45 control and GD 56 deficient groups) were randomized, sectioned sagittally into 5-μm-thick sections (Leica SM 2000R microtome; Leica Biosystems, Nussloch, Germany), and mounted on SuperFrost + slides (Thermo Fisher Scientific, Waltham, MA, USA). For the stereological calculations, the sections were selected from a random starting point with a distance of 400 μm between sections for GD 45 brains and 600 μm between sections for GD 56 brains, achieving a frequency of approximately 5 to 8 slides per hippocampus. The slides were deparaffinized and incubated in batches with TB3 antibody (1:2000, mAB 1637, Merck Millipore, Billerica, Massachusetts, USA) overnight at 4 °C in a randomized order. Control sections were included to correct for differences in staining intensity between batches. As a control for the specificity of antibody binding, IgG1 (1:200,