Prenatal vitamin C deficiency results in differential levels of oxidative stress during late gestation in foetal guinea pig brains

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**ABSTRACT**

Antioxidant defences are comparatively low during foetal development making the brain particularly susceptible to oxidative stress during antioxidant deficiencies. The brain is one of the organs containing the highest concentration of vitamin C (VitC) and Vit C deficiency during foetal development may place the brain at risk of redox status imbalance. In the present study, we investigated the developmental pattern and effect of VitC deficiency on antioxidants, vitamin E and superoxide dismutase (SOD), assessed oxidative damage by measuring malondialdehyde (MDA), hydroxynonenal (HNE) and nitrotyrosine (NT) and analysed gene and protein expression of apoptosis marker caspase-3 in the guinea pig foetal brain at two gestational (GD) time points, GD 45/pre-term and GD 56/near term following either a VitC sufficient (CTRL) or deficient (DEF) maternal dietary regime. We show that except for SOD, antioxidants and oxidative damage markers are differentially expressed between the two GDs, with high VitC (p < 0.0001), NT modified proteins (p < 0.0001) and active caspase-3 levels (p < 0.05) at pre-term and high vitamin E levels (p < 0.0001), HNE (p < 0.0001) and MDA (p < 0.0001) at near term. VitC deficiency significantly increased SOD activity (p < 0.0001) compared to CTRLs at both GDs indicating a compensatory response, however, low levels of VitC significantly elevated MDA levels (p < 0.05) in DEF at near term. Our results show a differential regulation of the investigated markers during late gestation and suggest that immature brains are susceptible to oxidative stress due to prenatal vitC deficiency in spite of an induction of protective adaptation mechanisms.

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**Introduction**

The developing brain has a high metabolic activity making it vulnerable to oxidizing agents such as free radicals [55], a situation that is further exacerbated by a yet immature scavenging antioxidant system in early life [33,48]. Although antioxidant defence mechanisms in guinea pig brains during foetal development have been investigated [36], the manifestation of macromolecular modifications due to lipid peroxidation or by similar oxidizing agents and cell death in the near term brain is unknown. It is also not known if the pertinent oxidative changes during development are likely to increase in the brain when deprived of optimal antioxidant levels.

Guinea pigs are precocial rodents with a gestation length of approximately 66 days with the peak of exponential brain growth ("the brain growth spurt") being reached about 15 days before birth (i.e. around gestation day (GD) 50) [12]. Interference with optimal requirements for the foetus may impose several negative effects on developmental outcome such as lower body weight [11] and in the brain lead to reduction of cells in hippocampal morphogenesis [4] and cognitive alterations as observed in rats [38]. Oxidative stress caused by deficiency in antioxidants like glutathione in the developing brain has been shown to negatively affect mitochondria [21] and also to result in an age-dependent neurodegeneration in the hippocampus [1].

Activity of antioxidant enzymes in guinea pigs such as superoxide dismutase, catalase, glutathione peroxidase (GPx) and glutathione reductase have been demonstrated to remain constant from GD 30 to 45 and to increase from GD 45 to GD 60, with the exception of superoxide dismutase (SOD) that remained constant throughout the GD 30–60 period [36]. In rats, postnatal GPx activity in the brain has been found to remain constant from birth...
whereas the activity of cytoplasmic SOD increases from birth until 60 days after birth in rats [34]. Analysis of antioxidant protein levels in developing mice brains from embryonic day 18 to postnatal day 21 has shown that total SOD activity, catalase and GPx activity increases suggesting an increase in antioxidants in perinatal and neonatal brains relative to foetal development [25]. Thus, despite inter-species differences and differences in degree of postnatal development (i.e. altrician vs. precocial species), there seems to be a general trend of increasing antioxidant capacity in the brain during the course of development. Hence, a decrease in antioxidant capacity could potentially disturb the redox homeostasis in the brain leading to negative consequences on development.

Vitamin C (VitC) deficiency in humans has been associated with increases in premature births and predisposing newborns’ to oxidative stress [39,44]. VitC is one of the primary antioxidants in the brain and selectively accumulates in high amounts during foetal development [14,26,33]. Interruption of vitC transport to the brain in the svct2−/− mouse is detrimental to perinatal survival [13,50], underlining the pivotal role of vitC in the developing central nervous system. Due to its one electron reduction potential, VitC effectively inhibits lipid peroxidation and scavenges several reactive oxidizing compounds such as superoxide, hydrogen peroxide and hydroxyl radicals as well as enabling the recycling of other antioxidant compounds such as vitamin E [7,20,30,49]. In guinea pigs, VitC deficiency during both pre- and postnatal development has been linked to deviations in hippocampal development [51,52]. Alteration of apoptotic mechanisms due to redox imbalance in the foetal brain has also been reported in several studies, however, the underlying mechanism and potential cause vs consequence remains to be disclosed [19,43].

Like humans, guinea pigs cannot synthesize VitC due to a non-functional gulonolactone oxidase (Gulo) gene [40] and therefore rely on an adequate dietary supply. The present study investigated the developmental course of and potential effects of VitC deficiency on markers of antioxidants, redox imbalance and apoptosis signalling in foetal guinea pig brains at two different gestational time points at which brain growth is at a peak, GD 45/pre-term and GD 56/near term.

Materials and methods

Animal experiment

The animal study adheres to the guidelines of EU Directive 2010/63/EU and was approved by Danish Animal Experimentation Inspectorate. Twenty pregnant guinea pigs between GD 6–10 were obtained from Charles Rivers Lab, Kieslegg, Germany. The animals were microchipped subcutaneously (PET-CHIP ID, Danworth farm, West Sussex, UK) and randomized according to GD and body weight into two groups receiving diets only differing in VitC content (specialized diets sniff, GmbH). Control: CTRL (900 mg/kg diet, n = 10) and Deficient: DEF (100 mg/kg diet, n = 10). It has previously been shown by us that the 100 mg VitC diet in guinea pigs results in non-scorbutic VitC deficiency [32,53]. Each of the groups was further randomized to having caesarian section (followed by euthanasia) performed at GD 45 or GD 56. The animals were housed in floor pens with straw bedding and feeding, hay and water were provided ad libitum. They were weighed once every week and blood was sampled (~300 μl) once in every two weeks from v. saphena at its superficial course on tibia to verify VitC status (data not shown).

At euthanasia three dams, one from CTRL group and two from DEF group were found not to have conceived. Necropsy revealed no signs of underlying disease and the animals were excluded from the study.

Euthanasia

Caesarean section was conducted on dams at GD 45 or GD 56. Ten to fifteen minutes prior to anaesthesia, dams were injected with 2 mg/kg body weight Torbugesic (10 mg/ml butorphanol, Scan Vet Animal Health, Fredensborg, Denmark) subcutaneously to achieve analgesia. Anaesthesia was achieved by inhalation of isofluran (Isoba Vet 100%, Intervet International, Boxmeer, The Netherlands). After the disappearance of voluntary reflexes (interdigital and skin-pinch), caesarean was performed by laparotomy through linea alba exposing the uterus. Excision of fetuses was done one at a time starting from the apex of the left horn towards the basis and subsequently commencing at the apex of the right horn. Immediately following delivery of each pup, the body weight was recorded, an intracardial blood sample was taken and the pup was euthanized by decapitation, the procedure lasting no more than 2 minutes. In the event of a pup displaying reflexes, euthanization by intraperitoneal injection of 0.5 ml pentobarbital (200 mg/ml) supplemented with lidocaine (Veterinary Pharmacy, University of Copenhagen, Denmark) was performed. Gender was recorded and post mortem autopsy with tissue sampling was performed on each pup, tissues allocated either to fixative or frozen for later analysis. Blood samples were centrifuged, stabilized and frozen after the intracardial blood sampling of the final pup from each dam. Once all the pups were removed from the uterus, thoracotomy of the dam was performed and an intracardial blood sample was taken before sacrificing by decapitation and exsanguination.

The brain was removed and the left hemisphere frozen in liquid nitrogen and the right hemisphere fixated in 4% PFA (paraformaldehyde in phosphate buffered saline, 0.15 M, pH 7.5) for 48 h then transferred to 1%PFA for long term storage. All frozen tissues were stored at −80 °C until further analysis. For the current study all foetal left brain hemispheres from a total of 85 fetuses, were blocked for gender and body weight and randomized to be used for gene and protein expression analysis (N values; 12 CTRL/GD 45, 9 DEF/GD 45, 10 CTRL/GD 56, 11 DEF/GD 56) or biochemistry (N Values; 12 CTRL/GD 45, 10 DEF/GD 45, 10 CTRL/GD 56, 11 DEF/GD 56).

Biochemistry

Analysis of VitC and malondialdehyde (MDA) in brain were performed as described previously [28,29,31]. Briefly, tissue samples (app. 0.5 g) were homogenized in PBS, centrifuged at 16,000 x g for 1 min at 4 °C. For VitC analysis, an aliquot was stabilized with an equal volume of 10% meta-phosphoric acid containing 2 mM EDTA (Merck, Whitehouse Station, NJ, USA), centrifuged, and the supernatant analysed by high-performance liquid chromatography (HPLC) with colorimetric detection. Levels of MDA were assessed by thiobarbituric acid derivatization followed by specific quantification of the genuine MDA(TBA)2 adduct by HPLC with fluorescence detection.

Analysis of α-tocopherol and γ-tocopherol was performed by HPLC with coulometric detection as modified from Sattler et al. [46]. Briefly, to 100 μl of tissue homogenate was added 25 μl freshly prepared 2,6-di-tert-butyl-p-cresol (10 mg/ml; Sigma, Copenhagen, Denmark), 100 μl sodium dodecyl sulphate (29 mg/ml, Sigma), 800 μl H2O, 900 μl ethanol and 100 μl 2-propanol. The cold mixture was extracted with 1 ml of n-hexane (Merck, Damstadt, Germany) of which 500 μl of organic phase was reduced to dryness at 40 °C using an airstream and subsequently redissolved in 100 μl ethanol for 2 min using a vortex mixer. Following centrifugation, 20 μl of the supernatant was used for HPLC analysis. Superoxide dismutase activity (SOD) was analysed using the Ransod colorimetric assay (SD125, Randox Laboratories Limited, UK) on tissue lysates according to manufacturer’s instructions.
RNA extraction and RT-PCR

Approximately 25 mg of each of brain tissues was homogenized in trizol (Invitrogen, Merelbeke, Belgium) and precipitated with chloroform (Sigma Aldrich, Steinheim, Germany) and isopropanol (Merck, Darmstadt, Germany). Purified RNA (SV Total RNA Isolation System, Promega, Madison, WI, USA) was eluted with 50 μl nuclease free water and the purity of RNA was determined by absorbance ratios A260/A280 and A260/A230 (Nanodrop 2000; Thermo Scientific, Wilmington, DE, USA). cDNA synthesis was performed by RT-PCR with 2 µg of RNA (MmLV RT enzyme, 5 × MmLV buffer and RNasin (Promega)); 10 mM dNTPs and Oligo (dT) primers (60 µg/120 µl) (Fermentas GmbH, St Leon Roth, Germany); Random hexamer primer (2 µg/µl) (GE Healthcare, Uppsala, Sweden).

Gene expression analysis

Intron-spanning beta-actin primers were used on all CDNA (Table 1) prior to real time quantitative PCR (Q-PCR) to test for genomic contamination. None of the included samples displayed signs of contamination. PCR products of included genes were run on 2% agarose gels to confirm specificity. Gene expression analysis of Caspase 3 from brain samples was normalized to the reference gene, s18 (ribosomal protein S18). Primers are listed in Table 1.

Protein extraction and Western blot

Protein was extracted from brain tissue with radio-immuno-precipitation assay buffer (RIPA: 150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0% sodium dodecyl sulphate, 25 mM Tris, pH 8) with 1:100 protease inhibitors (Sigma complete protease inhibitor cocktail). Estimation of protein was done by bichrocinic acid assay (Calbiochem Novagen BCA Protein Assay kit), according to the manufacturer’s protocol, in triplicates (in dilution 1:5), nuclease free water as negative control and calibrator as positive control. Gel to PVDF transfer unit (TE 77PWR, Amersham Biosciences) prior to 1 h nuclease free water as negative control and calibrator as positive control. Gel to PVDF transfer unit (TE 77PWR, Amersham Biosciences) prior to real time quantitative PCR (Q-PCR) to test for genomic contamination. None of the included samples displayed signs of contamination. PCR products of included genes were run on 2% agarose gels to confirm specificity. Gene expression analysis of Caspase 3 from brain samples was normalized to the reference gene, s18 (ribosomal protein S18). Primers are listed in Table 1.

Protein was extracted from brain tissue with radio-immuno-precipitation assay buffer (RIPA: 150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50 mM Tris, pH 8) with 1:100 protease inhibitors (Sigma complete protease inhibitor cocktail). Estimation of protein was done by bichrocinic acid assay (Calbiochem Novagen BCA Protein Assay kit), according to the manufacturer’s protocol, in triplicates (in dilution 1:5), nuclease free water as negative control and calibrator as positive control. Gel to PVDF transfer unit (TE 77PWR, Amersham Biosciences) prior to real time quantitative PCR (Q-PCR) to test for genomic contamination. None of the included samples displayed signs of contamination. PCR products of included genes were run on 2% agarose gels to confirm specificity. Gene expression analysis of Caspase 3 from brain samples was normalized to the reference gene, s18 (ribosomal protein S18). Primers are listed in Table 1.

Statistics

SAS/JMP statistical version 8.0 was used to analyse all data. Analysis of Q-PCR expression data was performed by Student’s t-test. Two-way ANOVA was used to analyse biochemistry and Western blot densitometry. Interaction between factors, diet and GD, was reported together with main effects when statistically significant (p < 0.05) followed by Tukeys HSD posthoc comparisons.

Results

Brain antioxidant status

As shown in Table 2, VitC concentration in the brain was significantly higher in the CTRL groups of both gestational days compared to DEF counterparts (p < 0.0001). Two-way ANOVA showed effects of both diet and GD (p < 0.0001) on VitC levels as well as an interaction between diet and GD (p < 0.0001). A decrease of approximately 20% in VitC was observed in CTRL GD 56 relative to GD 45 (p < 0.0001). This was not found in the DEF group, where VitC levels did not differ between gestational dates. In contrast to total VitC in the brain, α-tocopherol levels were high on GD 56 relative to GD 45 (p < 0.0001) with no significant effect of diet. Conversely, two-way ANOVA showed a significant effect of diet on γ-tocopherol (p < 0.05), the DEF-group displaying overall increased levels. However, tocopherols were not significantly different between CTRL and

Table 1

| Gene       | Primer sequence (bp) | Product size (bp) | NCBI accession no. |
|------------|----------------------|-------------------|--------------------|
| Beta-actin | (F): gtagaacctctatgcaccaca 346 | AF508792          |                    |
| s18        | (F): atgctcaacctctgctttct 195 | XM_003473925.1    |                    |
| Caspase-3  | (F): ccggagaaggctcgatt 315 | XM_003469171.1    |                    |

Sequences are presented in the 5’-3’ direction (F): forward, (R): reverse.
In agreement with the anti-HNE densitometry data, MDA was significantly lysate and almost all bands in the positive control antibody with free NT abolished all the bands in guinea pig brain between 35 and 15 kDa in brain lysates. Pre-absorption of primary antibody with blocking peptide abolished the specific bands from guinea pig brain lysate and the positive control confirming the specificity of the antibody (data not shown). Two-way ANOVA of densitometry data showed that GD had a significant effect on cleaved caspase-3 (p < 0.05) with higher levels at GD 45 compared to GD 56 (Fig. 2B). Densitometry analysis at either gestational day did not show significant differences between CTRL and DEF groups (Fig. 2B).

**Table 2**

| Brain antioxidant status in prenatally VitC deficient and control guinea pigs at GD 45 and GD 56. | CTRL GD 45 | CTRL GD 56 | DEF GD 45 | DEF GD 56 | Effect of VitC | Effect of GD |
|---|---|---|---|---|---|---|
| VitC (nmol/g tissue) | 3043 ± 285a | 2351 ± 177b | 1016 ± 270c | 966 ± 242d | *** | *** |
| α-Tocopherol (nmol/g tissue) | 5.5 ± 0.8a | 9.0 ± 1.0a | 4.6 ± 1.0b | 9.4 ± 1.5c | NS | *** |
| γ-Tocopherol (nmol/g tissue) | 0.52 ± 0.46 | 0.50 ± 0.36 | 0.80 ± 0.5 | 0.89 ± 0.39 | * | NS |
| Superoxide dismutase (U/g tissue) | 90 ± 2a | 92 ± 2a | 133 ± 3b | 137 ± 4c | *** | NS |

CTRL: controls; DEF: deficient; GD: gestational day. Values are presented as means ± SD. Differences between groups were assessed using two-way ANOVA with diet and GD as factors followed by Tukeys HSD test for individual comparisons. NS: not significant. Different superscript letters indicate that groups are significantly different.

\*p < 0.05.
\*\*p < 0.001.
\*\*\*p < 0.0001.

**Fig. 1.** Oxidative damage in foetal guinea pig brains. Markers of increased oxidation and suggestive oxidative damage in the brain of guinea pigs subjected to prenatal VitC deficiency (DEF) (dark bars) or control diet (CTRL) (light bars) during development. Foetuses were excised by caesarean section at GD 45 and GD 56. (A) Densitometric analysis of HNE modified proteins in brain homogenate; results are normalized to actin; (B) Malondialdehyde in brain homogenate, measured by HPLC; (C). Densitometric analysis of nitrotyrosine modified proteins normalized to actin. Data are expressed as means ± SD. \*p < 0.05; \*\*p < 0.001 by ANOVA.

**Discussion**

In the present study, we show that immature brains are susceptible to oxidative stress in spite of an induction of protective adaptation mechanisms. Oxidative stress and damage markers in guinea pig cerebral cortex were assessed at preterm and near term gestational time points with and without maternal VitC deficiency as a potential oxidative insult.

Brain VitC levels in DEF groups were significantly lower than CTRL as expected, and while VitC levels in the DEF group remained similar at the two gestational days, VitC in GD 56 CTRL was significantly lower than GD 45 CTRL. This finding indicates a developmental requirement of increased VitC during the preterm period possibly as a response to lower protective mechanisms against oxidizing agents as has previously been suggested [36]. However, in contrast to CTRLs, the DEF group did not display a near-term drop in concentration but rather showed retention of VitC, possibly to avoid any further loss than the already existing low level. The decrease in VitC toward term in CTRL animals is in line with findings in humans reporting a late gestation decrease of VitC in all foetal tissues including the brain [58,59]. This decrease in the brain has been suggested to be due to growth
and maturation of non-neuronal cells and maturation and myelination of neurones [16,59]. Recent studies by us have shown that a pre-natal low VitC causes a reduction in foetal body and brain weight at GD 45 but not at GD 56 (unpublished results), indicating that a developing foetus is able to compensate for a pre-imposed negative effect of VitC deficiency during the final weeks of gestation.

Our α-tocopherol data implies that it increases with progression in development during late gestation. Interaction between cytosolic VitC and membrane bound vitamin E regenerates oxidized vitamin E and results in an efficient antioxidant mechanism [9], which suggests that VitC deficiency may alter vitamin E levels hereby possibly disrupting membrane integrity. Some in-vivo studies have shown that intake of high VitC results in an increased vitamin E in tissues [5,17,18]. This was not the case when evaluating α-tocopherol in CTRL vs. DEF groups of this study, however, VitC showed a main effect on γ-tocopherol levels in the brain. γ-Tocopherol has been associated with anti-inflammatory properties, decreased superoxide anion generation and inhibition of lipid peroxidation, hereby reducing adverse effects of oxidative imbalance [23,45]. Our present findings suggest an increase in γ-tocopherol in response to VitC deficiency, whereas levels during late gestational progression were relatively constant in both CTRL and DEF groups.

In the current study, VitC deficiency increased SOD activity during late gestation. SOD activity in CTRL groups at both GD is in agreement with a previous report of constant SOD levels in guinea pig foetal brains throughout gestation from GD 30 to GD 60 [36]. As a consequence of low VitC levels, increases in SOD activity in DEF compared to CTRLs at both GD indicates foetal ability to induce a compensatory mechanism, likely due to increased production of superoxide radicals [24]. Various studies have reported an increase in SOD activity in the brain as an adaptive mechanism to tolerate oxidative damage in neurodegenerative conditions [41], stress [10] and oxidant induced neurotoxicity [6]. Studies like these with additional assessment of antioxidants like catalase, glutathione peroxidase or oxidative damage markers helps in understanding whether such adaptive mechanisms are able to preserve the redox status [3,57].

HNE modified proteins, which indicate oxidative damage due to lipid peroxidation, were significantly high at GD 56, regardless of VitC status, likely due to the increase in poly-unsaturated fatty acids in the brain at near term. An increased susceptibility of lipid peroxidation in near term brains of guinea pigs has previously been reported [37]. Although HNE modified proteins were not significantly different between CTRL and DEF, a trend similar to the obtained MDA levels was observed between the two GD, with significantly higher MDA levels at near term further supporting the maturational related increase of lipid peroxidation in the brain as reported by others in guinea pigs brain [37]. Significant increase of MDA levels in DEF at GD 56 and not in DEF at GD45 compared to their CTRL suggests that compensatory mechanisms that may have been present at earlier gestation may not be enough to protect from lipid peroxidation due to low levels of VitC in the brain. Earlier studies by us and others have shown a similar inverse relation of VitC levels to lipid peroxidation in the brain [15,33] and the vital role of ascorbate has previously been demonstrated to inhibit lipid peroxidation in rat brain microsomes [47]. Conversely, no significant differences in NT modified proteins between CTRL and DEF suggests that posttranslational modification of proteins by NT is not affected by VitC deficiency and instead, as proposed by others, NT may be involved in mediating cell signalling at its basal levels during development [27,35].

Results from oxidative stress based in-vivo studies have suggested an increased free radical production or decreased antioxidant mechanisms in the immature brain [36,56], which in turn may initiate cellular events to initiate mechanisms of apoptosis [8,42] like caspase-3 activation [22]. Our finding of high levels of cleaved caspase-3 in GD 45 compared to GD 56 indicates a developmental phenomenon in agreement with what has been shown by others [2], placing cleaved caspase-3 as playing an important role in reducing neuronal overproduction in the developing brain [54]. The guinea pig is a precocial species and neuronal number relative to adult stage are achieved by GD 48 [12]. Hence, although a gestational effect was seen for cleaved caspase-3, the difference was small implying that most of the cell death associated with development is likely to have occurred before the investigated time-points. No significant differences in caspase-3 gene expression or cleaved caspase-3 protein levels between CTRL and DEF groups at any of the GDs indicates that VitC deficiency does not modulate caspase-3 mediated apoptosis and may not be the underlying mechanism associated with hippocampal impairment observed in our earlier findings [51,52].

**Conclusion**

Guinea pig foetal brains have differential requirements of antioxidants with high VitC levels at pre-term, a higher requirement of α-tocopherol at near term and constant SOD activity. Oxidative NT modifications and active caspase-3 levels are higher at pre-term and are prone to increased lipid peroxidation at near term.
term significantly decreasing expression of oxidative damage markers associated with brain maturity. Prenatal VitC deficiency in the guinea pig foetal brain does not modulate levels of vitamin E, NT or HNE protein modifications or caspase-3 however, increases SOD activity as compensation although this is not adequate to prevent increased lipid peroxidation at the investigated time points.

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