ORIGINAL ARTICLE

Mitochondrial Dysfunction, Oxidative Stress and Premature Aging in Children With Nutritional Rickets

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ABSTRACT

Objective: To assess the mitochondrial dysfunction, oxidative stress and premature aging in children with nutritional rickets.

Methods: This cross-sectional study enrolled children between 6 months - 5 years of age with nutritional rickets attending a tertiary care hospital between between January 2021 and August 2022. Mitochondrial dysfunction, oxidative stress and premature aging were assessed by measuring the mitochondrial DNA (mtDNA) content, total antioxidant status (TAOS) and telomere length (TL) in 40 children with nutritional rickets and 40 age- and sex- matched healthy children without rickets (controls).

Results: The median (IQR) mtDNA content was significantly higher in children with rickets as compared to controls [152.27 (111.83,218.66) vs 93.7 (72.5,134.14); $P < 0.001$], implying mitochondrial dysfunction attributed to increased mitochondrial biogenesis in children with rickets. The median (IQR) TAOS was significantly lesser in children with rickets than controls [4.54 (3.93, 5.73) vs 7.86 (5.09, 9.58); $P < 0.001$)]. The median (IQR) TL in cases was significantly longer in children with rickets compared to controls [417.31 (111.83,218.66) vs 93.7 (72.5,134.14); $P < 0.001$] implying that children with rickets do not have premature aging.

Conclusion: Children with rickets have high oxidative stress and mitochondrial dysfunction but no evidence of premature aging.

***Keywords*:** Antioxidant, Cell death, Mitochondrial DNA, Telomere length, Vitamin D deficiency

INTRODUCTION

Vitamin D exerts a protective effect on the cellular machinery including mitochondria through various mechanisms. One of these is the effect of vitamin D on mitochondrial respiratory activity that limits the generation of reactive oxygen species (ROS) [1,2]. Vitamin D deficiency (VDD) in disease conditions like lower back pain, multiple sclerosis, and diabetic vasculopathy, also leads to increased generation of ROS which in turn leads to mitochondrial dysfunction [3,4]. VDD has also been associated with an increased oxidative stress and inflammation in children and adolescents.

Mitochondrial DNA (mtDNA) content in bodily fluids serves as an indicator of mitochondrial dysfunction. Mitochondrial to nuclear (Mt/N) DNA ratio may be a biomarker for mitochondrial dysfunction and an increase in Mt/N DNA ratio may precede mitochondrial impairment as an adaptive response and is a predictive biomarker for mitochondrial dysfunction [5]. Telomere length (TL) is used as a standard biomarker for premature aging which is characterized by telomere shortening [6]. Antioxidants and ROS have opposite effects on TL and the link between chronic oxidative stress (OS) and telomere shortening is supported by both in vitro and in vivo experimental findings. Vitamin D levels have shown positive correlation with TL and also decreased senescence and apoptosis [7,8].

Mitochondrial dysfunction and premature aging have not been studied in children with VDD states like rickets. We, therefore, planned this study to see the mitochondrial dysfunction, oxidative stress and premature aging in children with nutritional rickets and compare it with healthy controls. MtDNA content, total antioxidant status (TAOS) and TL were used as markers of mitochondrial dysfunction, oxidative stress and premature aging, respectively.

METHODS

This cross-sectional study was conducted between January 2021 and August 2022 in the Departments of Pediatrics and Biochemistry of a tertiary public hospital affiliated to a medical college in Delhi, India. An approval from Institutional Ethics Committee of the institute and a written informed consent from the parents/guardians of the participating children were obtained.

Children aged 6 to 59 months attending the pediatric outpatient department with clinical evidence of rickets (wide wrists, bow legs, frontal bossing, rachitic rosary) and radiological findings consistent with a diagnosis of nutritional rickets (fraying, splaying, and cupping at the metaphyseal ends of long bones in wrist/knee) were enrolled (cases) [9,10]. Age-and sex-matched children who presented to the pediatrics department with minor illness (e.g., upper respiratory tract infection) without any signs of rickets were enrolled as the comparison (control) group. Children with comorbid chronic systemic diseases (renal, cardiac, hepatic), height less than -3SD, skin diseases, known cases of skeletal dysplasia, hypophosphatemic and vitamin D dependent rickets and those on vitamin D supplements were excluded from the study. A convenient sample size of 40 in each group was planned, considering that no prior work is available on mitochondrial dysfunction, oxidative stress and premature aging in children with nutritional rickets.

A detailed clinical history was taken and a thorough physical examination was performed at the time of enrolment. Exposure to sunlight was a rough approximation assessed by interviewing the parents. Calorie intake was estimated using 24-hour dietary recall method. Assessment of weight (digital weighing scale, sensitivity 5 g; SECA), length/height (infantometer/stadiometer, sensitivity 1 mm; Seca) and mid upper arm circumference (MUAC) was done as per standard methods [11]. Severity of radiological rickets was assessed using Thacher score [12]. WHO Child Growth Standards were used as reference [13]. The WHO Anthro software for PC [14] was used to calculate z-scores for all anthropometric parameters. Children with rickets were managed as per the Indian Academy of Pediatrics (IAP) recommendations for vitamin D and calcium deficiency [9].

Venous blood samples were obtained before starting treatment of rickets with vitamin D and calcium. Blood was collected in EDTA vial (1 mL) and plain vial (2 mL) and stored in aliquots at – 80°C. EDTA blood was analysed for mtDNA content and TL, and serum was analysed for 25-hydroxy vitamin D (25OHD), TAOS, serum calcium, phosphorous, alkaline phosphatase, liver and kidney function tests.

DNA extraction was done using a commercially available column-based kit (QIAamp DNA Blood Mini Kit, Qiagen). It was quantified using a spectrophotometer (NanoDrop 2000c). 50 ng of DNA was further used for assessment of TL based on the method described by Cawthon et al [15] with minor modifications by using multiplex real time polymerase chain reaction (PCR) (CFX ConnectTM Real Time System, BIO-RAD). Sequence of primers (Sigma) used are given in **Table I**. Telomere (T) PCR and a single

copy gene (S) i.e. β-globin gene PCR were performed as a multiplex PCR setup using dye (Evagreen, BIOTIUM) based chemistry (DreamTaq PCR Male Mix, Thermo Scientific). The cycle threshold (Ct) values thus obtained were used to determine the T/S ratio which was a measure of relative telomere length.

The relative mtDNA copy number was estimated by Real time PCR (CFX Connect Real Time System, Bio-Rad). Mitochondrial- and nuclear-specific primers were used in two separate set of PCR reactions. PCR was performed as a setup using dye-based chemistry (DreamTaq PCR Male Mix, Thermo SCIENTIFIC). The ratio of Ct values gave the relative mtDNA content, which in turn, reflected the mitochondrial copy number [16]. To determine the mtDNA content relative to nuclear DNA, the following equation was used:

ΔCt = (nucDNA Ct – mtDNA Ct) and Relative mitochondrial DNA content = 2 × 2ΔCt

Sequence of primers (Sigma) used are given in **Table II**.

Antioxidant assay kit (Cayman Chemicals) was used to measure the TAOS as per the manufacturer's instructions. The assay relies on the ability of antioxidants in the sample to inhibit the oxidation of 2,2'-azino-di-[3-ethylbenzothiazoline sulphonate] (ATBS) by metmyoglobin. The amount of ATBS produced was monitored by reading the absorbance at 750 nm. The capacity of the antioxidants in the sample to prevent ATBS oxidation was compared with that of Trolox (standard curve), a water -soluble tocopherol analogue, and was expressed as millimolar Trolox equivalents (mM Trolox).

Serum 25-OH vitamin D level was estimated in Beckman Coulter Access 2 immunoassay analyzer by chemiluminescence technique using commercially available standard kits. The cut-off for vitamin D status based on serum 25(OH) vitamin D levels were: deficiency < 12 ng/mL (30 nmol/L), insufficiency 12-20 ng/mL (30-50 nmol/L), sufficiency > 20 ng/mL (50 nmol/L), toxicity > 100 ng/mL (250 nmol/L) [9]. Serum calcium (mg/dL) was estimated using calcium arsenazo 111 as calcium analyte (UniCel DxC 600 automatic analyser, Beckman Coulter India Pvt. Ltd.). Hypocalcemia was defined as total serum calcium < 8.8 mg/dL. [17] Serum phosphate level was estimated using timed end-point method (UniCel DxC 600 automatic analyser, Beckman Coulter India Pvt. Ltd.). Hypophosphatemia was defined as serum phosphate < 3.8 mg/dL. [17] Serum alkaline phosphatase (ALP) level was estimated by pNPP (p-nitrophenyl phosphate) method (UniCel DxC 600 automatic analyser, Beckman Coulter India Pvt. Ltd.). Raised alkaline phosphatase was defined as level > 420 IU/L. [17]

Statistical analysis: Categorical data were expressed as proportions. Continuous data like mtDNA content, TL, TAOS and 25OHD levels were expressed as mean (SD) or median (IQR). Comparison of mtDNA content, TL and TAOS between the groups was done using unpaired Student *t*-test or Mann-Whitney U test. Correlation between 25OHD level and mtDNA, TL, and TAOS and that between TL and mtDNA content was done using Karl Pearson's correlation coefficient. The level of significance was taken as $P < 0.05$.

RESULTS

A total of 40 children (15 girls) with rickets with a mean (SD) Thacher score of 3.6 (1.66) and 40 age-and sex-matched controls were enrolled. The mean (SD) age of the study participants was 29 (13.3) months. Each group had 3 (7.5%) children below 12 months, 11 (27.5%) between 12-24 months and the remaining above 24 months. Nineteen (47.5%) children with rickets, presented with poor growth, 16 (40%)

had wrist widening, 12 (30%) had recurrent chest infections and 9 (22.5%) had limb deformities. On examination, 39 (97.5%) had wrist widening, 32 (80%) had frontal bossing, 16 (40%) had protuberant abdomen, 11 (27.5%) had delayed anterior fontanelle closure and 9 (22.5%) children had bony deformities. **Table III** compares the baseline characteristics and vitamin D status of study groups; severe stunting or wasting was not observed in any participant. In children with rickets, hypocalcemia was found in 34 (85%), low serum phosphorous in 4 (10%) and raised serum alkaline phosphatase in all cases. In controls, 10 (25%) had low serum calcium, 5 (12.5%) had low serum phosphorous and 2 (5%) had elevated serum alkaline phosphatase levels. 30 (75%) out of 40 children with rickets had vitamin D deficiency (<12 ng/mL). Remaining 10 (25%) had insufficient (12-20 ng/mL) levels. 29 (72.5%) out of 40 controls had insufficient, 7 (17.5 %) had sufficient and 4 (10 %) had deficient vitamin D levels. In our study, although the cases and controls did not have severe underweight, stunting and wasting; cases had significantly lower weight-for-age, height/length-for-age and weight-for-height/length z-scores as compared to controls.

Table IV compares the mtDNA content, TAOS and TL between cases and controls which were significantly different. The median (IQR) mtDNA content of cases was significantly higher ($P < 0.001$) than controls implying mitochondrial dysfunction in children with rickets. The antioxidant capacity measured by TAOS was also significantly lesser in cases ($P < 0.001$). Telomere length was significantly longer in cases compared to controls ($P < 0.001$). The outcome variables were also calculated after adjusting the anthropometric parameters (**Table V**).

The mtDNA content and TL had statistically significant negative correlation with vitamin D level ($r = - 0.247$, $P = 0.027$) and ($r = - 0.252$, $P = 0.024$), respectively in all the participants ($n = 80$). The correlation between TAOS and vitamin D level was statistically insignificant ($r = 0.195$, $P = 0.083$). The mtDNA content, TAOS and TL in children with rickets had no significant correlation with vitamin D levels (r = - 0.015, $P = 0.926$; r = -0.117, $P = 0.474$; r = - 0.010, $P = 0.949$), respectively]. Also, the severity of rickets (as measured by Thacher score) did not correlate with mtDNA content (r = - 0.084, P = 0.604), TAOS (r = 0.232, $P = 0.150$) and TL (r = 0.072, $P = 0.657$).

DISCUSSION

The present study shows lower antioxidant status, higher mitochondrial dysfunction and higher telomere length in children with rickets compared to controls. The increased oxidative stress is postulated to cause a compensatory increase in mitochondrial biogenesis and thus increased mtDNA content in children with rickets. TL was not reduced in rickets cases. Statistically significant negative correlation of vitamin D levels with mtDNA content and TL was found. This indicates that vitamin D deficiency leads to increase in mtDNA content and TL. Whereas, TAOS showed positive correlation with vitamin D level although this was statistically insignificant.

In the present study, TAOS was significantly reduced in children with nutritional rickets, showing that defense system against oxidative stress is impaired in VDD. This finding corroborates with other studies showing an increased oxidative stress in VDD. A systematic review in children and adolescents found that poor vitamin D status is associated with oxidative stress and inflammation in children and adolescents [18].

Oxidative stress in turn causes mitochondrial dysfunction, which can be detected by mtDNA content. The mtDNA content in body fluids may be used as biomarker of mtDNA dysfunction [5]. The present study, observed higher mtDNA content in children with rickets. This is attributed to adaptive increase in mtDNA content in rickets.

As evidenced from a previous study, TL, mtDNA and TAOS are significantly different in children with SAM compared to those without SAM [20]. However, even after adjusting for the anthropometric differences between both groups, TL, mtDNA content and TAOS, were significantly different between rickets cases and healthy controls.

A dose-response correlation between TL and 25OHD has been reported, with the likelihood of having a relative higher TL than the median, rising with higher levels of 25OHD [21]. However, unlike the above-mentioned studies, this study showed an increase in TL with decrease in vitamin D level and increase in oxidative stress. The longer TL in rickets cases as compared to controls in our study may have been due to the presence of hypocalcemia in 85% of cases as compared to 25% in controls. A study by O' Callaghan et al showed negative correlation between serum calcium level and telomere length.[21]. Earlier studies in adults and elderly have shown inconsistent association between TL and bone health as assessed by bone mineral density (BMD) [22-25], although we did not measure the BMD in our study participants. Bekaert et al. and Valdes et al found that TL was positively correlated with bone mineral density (BMD) in elderly males and adult females, respectively [22,23]. On the other hand, Sanders et and Tang et al found no association between TL and BMD [24,25]. This implies that other unidentified factors may influence the association between the two parameters.

These parameters are suggestive of increased oxidative stress as indicated by decreased TAOS and increased mtDNA content in children with rickets in this study but no premature aging as measured by the TL. This indicates that rickets perhaps does not cause permanent damage to DNA and can be reversed by appropriate and timely treatment. However, this study had small sample size with a cross-sectional design hence we did not assess the change in these parameters following vitamin D supplementation. Due to financial restrictions, we did not assess serum parathormone levels and BMD. Further, comparison group also had vitamin D deficiency/insufficiency, thereby limiting the subgroup analysis to ascertain the effect of VDD on TL, mtDNA content and TAOS.

To conclude, children with rickets have high oxidative stress leading to mitochondrial dysfunction. Telomere length was significantly high in cases implying no premature aging in children with rickets. Longitudinal studies with a larger sample size and monitoring of all the parameters following vitamin D administration are needed to further the research in this area.

Ethics clearance: Institutional Ethics Committee, University College of Medical Sciences and Guru Teg Bahadur Hospital, Delhi, No. IEC-HR/PG/46/69. dated Dec 21, 2020.

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Competing interest: None stated.

WHAT THIS STUDY ADDS TO?

- Children with nutritional rickets have increased oxidative stress and mitochondrial dysfunction but no premature aging

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Table I Sequence of the Primers Used for Estimation of Telomere Length

Telg (telomere F)	ACACTAAggTTTgggTTTgggTTTgggTTTgggTTAgTgT
Telc (telomere R)	TgTTAggTATCCCTATCCCTATCCCTATCCCTATCCCTAACA
Hbgu (β-globin F)	CggCggCgggCggCgCgggCTgggCggCTTCATCCACgTTCACCTTg
Hbgd (β-globin R)	gCCCggCCCgCCgCgCCCgTCCCgCCggAggAgAAgTCTgCCgTT

F Forward primer, R Reverse primer

Table II Sequence of the Primers Used for Estimation of mtDNA Content

Mitochondrial primer (F)	CACCCAAgAACAgggTTTgT
Mitochondrial primer (R)	TggCCATgggTATgTTgTTA
Nuclear primer (F)	TgCTgTCTCCATgTTTgATgTATCT
Nuclear primer (R)	TCTCTgCTCCCCACCTCTAAgT

Mitochondrial primers tRNA-Leu (UUR); Nuclear primers β2-microglobulin; F Forward primer, R Reverse primer

Table III Comparison of Baseline Characteristics in Cases Children with Rickets and Controls

Parameters	*Cases (n = 40)*	*Controls (n = 40)*	*P value*
Birth weight [a] (g)	2532.5 (371.41)	2498.1 (360.83)	0.404
Gestational age[a] (wk)	37.9 (1.95)	37.7 (1.62)	0.458
IFA/calcium intake during pregnancy	15 (54)	20 (66)	0.622
Sunlight exposure ≥ 30 min	6 (15)	7 (17.5)	0.900
Dietary deficit	40 (100)	28 (70)	< 0.001
Anthropometry [a]			
Weight-for-age z-score	-1.61 (0.55)	-0.40 (0.60)	< 0.001
Height for-age z-score/ Length-for-age z-score	-1.74 (0.72)	-0.53 (0.72)	< 0.001
Weight-for-height z-score	-0.94 (0.73)	-0.17 (0.77)	< 0.001
Head circumference	-0.86 (0.49)	-0.14 (0.54)	< 0.001
Mid upper arm circumference	14.12 (0.86)	14.23 (0.90)	0.555
Serum calcium (mg/dL)	7.96 (0.70)	9.02 (1.20)	0.742
Serum phosphorous (mg/dL)	4.59 (0.60)	4.35 (0.51)	0.055
Serum alkaline phosphatase (IU/L)	774.68 (244.21)	282.23 (62.69)	0.031
Serum vitamin D status			
< 12 ng/mL	30 (75)	4 (10)	< 0.001
12-20 ng/mL	10 (25)	29 (72.5)	
> 20 ng/mL	0	07 (17.5)	

Data expressed as n (%) or [a]mean (SD)

Table IV Comparison of mitochondrial DNA Content, Telomere Length, Total Antioxidant Status in Children With Nutritional Rickets and Controls

Parameters	*Cases (n = 40)*	*Controls (n = 40)*	*P value*
mtDNA	152.27 (111.83, 218.66)	93.7 (72.5, 134.14)	< 0.001
TL (T/S)	417.31 (111.83, 218.66)	93.7 (72.5, 134.14)	< 0.001
TAOS (mM trolox equiv)	4.54 (3.93, 5.73)	7.86 (5.09, 9.58)	< 0.001

Data expressed as median (IQR)
mtDNA mitochondrial DNA content, TL Telomere length, TAOS Total antioxidant status

Table V Comparison of Mitochondrial DNA Content, Telomere Length, Total Antioxidant Status Adjusted for Anthropometry in Children with Nutritional Rickets and Controls

Parameters	*Geometric mean (95% CI)*		*Risk ratio (95% CI)*	*P value**
	Cases (*n* = 40)	Controls (*n* = 40)		
mitochondrial DNA content				
Unadjusted	156 (137, 178)	99 (89, 110)	1.58 (1.34, 1.87)	< 0.001
Adjusted	158 (136, 183)	97 (84, 114)	1.61 (1.25, 2.08)	< 0.001
Telomere Length				
Unadjusted	402 (349, 463)	195 (164, 232)	2.06 (1.65, 2.56)	< 0.001
Adjusted	413 (338, 504)	190 (155, 232)	2.17 (1.55, 3.04)	< 0.001
Total Antioxidant Status (mM Trolox equivalents)				
Unadjusted	4.62 (4.07, 5.26)	6.70 (5.68, 7.90)	0.69 (0.56, 0.85)	0.001
Adjusted	4.49 (3.72, 5.42)	6.90 (5.73, 8.31)	0.65 (0.48, 0.89)	0.007

Analysis of covariance (F-test); adjusted for the weight-for-age, height-for-age, and weight-for-height