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Original Article

Peadiatrics Section

Detection and Quantification of Free Radicals in Peroxisomal Disorders: A Comparative Study with Oxidative Stress Parameters

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ABSTRACT

Introduction: Free radicals have been thought to participate in pathogenesis of peroxisomal disorders.

Objective: The aim of the work is to detect free oxide radicals in blood of patients with peroxisomal disorders and to study their relation with various oxidative stress parameters.

Materials and Methods: Twenty patients with peroxisomal disorders and 14 age and sex matched healthy subjects were included in the study. Patients with peroxisomal disorders were subdivided according to diagnosis into peroxisomal biogenesis disorders and single enzyme deficiency. Oxidative stress was evaluated in both patients and control subjects by assessment of free radicals, malondialdehyde, nitric oxide metabolites and superoxide dismutase.

Results: There was increase in free radicals, malondialdehyde, nitric oxide metabolites in patients compared with control subjects. However, there was decrease in superoxide dismutase levels in patients compared with control subjects.

Conclusion: We concluded that there is excess free radicals production accompanied with decrease in antioxidant defenses in patients with peroxisomal disorders. These results strongly support a role of free radicals in the pathophysiology of peroxisomal disorders and strengthen the importance of oxidative stress phenomenon in peroxisomal disorders pathogenesis.

Keywords: Malondialdehyde, Nitric oxide, Superoxide dismutase

INTRODUCTION

Free radicals are physiologically formed due to oxygen consumption in different metabolic reactions [1]. The main sources of free radicals are mitochondria, endoplasmic reticulum and peroxisomes [2]. They are highly unstable species because of the presence of unpaired electrons in their structure. They only become stable by coupling with other molecules [2]. Free radicals are divided into reactive oxygen species (ROS) that is including superoxide anion radicals, hydroxyl radicals and hydrogen peroxide and reactive nitrogen species (RNS) that is including peroxynitrite and nitric oxide (NO) [1].

Superoxide dismutase (SOD) and catalase are antioxidant enzymes that protect against overproduction of free radicals [3]. Imbalance between antioxidant enzymes and free radicals production will result in free radicals toxicity. Free radicals toxicity will lead to lipid peroxidation and release of malondialdehyde (MDA) and these changes will result in a condition called “oxidative stress” [4]. Disturbance of SOD activity, increased NO and MDA concentrations are considered important oxidative stress markers [4]. Oxidative stress is now considered as a hallmark of many inflammatory inherited neurological disorders [5].

Peroxisomes are these ubiquitous organelles that play a key role in both the production and scavenging of ROS in the cell due to their production of free radicals that results from high peroxisomal consumption of oxygen and presence of ROS-metabolizing enzymes in them [6]. Peroxisomes also catalyze variety of metabolic reactions especially beta-oxidation of VLCFAs (very long chain fatty acids) and alpha-oxidation of phytanic acid [7].

The peroxisomal disorders are usually subdivided into two groups. The first group is including the peroxisome biogenesis disorders (PBDs) which comprises Zellweger syndrome (ZS), neonatal adrenoleukodystrophy (NALD), infantile Refsum disease (IRD) and rhizomelic chondrodysplasia punctata (RCDP) type 1[8]. The second group is including the single peroxisomal enzyme (transporter) deficiency disorders which comprises atleast 12 different diseases, with Refsum disease and X-linked adrenal leukodystrophy (XALD) being the most common [4]. Patients with disorders in peroxisomal metabolism are suffering from progressive central nervous system demylination and marked motor and mental disabilities [8]. Elevation of VLCFA levels in the form of C26:0 levels, C24/C22 and C26/C22 ratios are consistent with peroxisomal fatty acid metabolism disorders [9]. It is speculated that accumulation of VLCFA in peroxisomal disorders patients may be associated with oxidative stress and overproduction of free radicals, so therapeutic plans that are directed to decrease accumulation of VLCFA, reduce ROS production and increase anti-oxidant enzymes concentration and activity will significantly improve disease symptoms and quality of life in these patients [10].

According to our knowledge, very limited studies measured free radicals and their relations to oxidative stress parameters in blood of patients with peroxisomal disorders especially in Egyptian patients.

This study aimed to detect free radicals in the blood of patients with peroxisomal disorders before de-oxidization by a suitable antioxidant, and to study their relation with various oxidative stress parameters.

MATERIALS AND METHODS

Materials

Considering peroxisomal disorders as very rare genetic diseases, all cases entered the outpatient genetics clinic of Cairo University through a time period extending from July 2014 till April 2015, were included in the study.

Twenty patients were selected from outpatient genetics clinic; Cairo University and were referred to National Research Centre for further

investigations. All patients suffered from peroxisomal disorders. There were fifteen males (75%) and five females (25%). Their mean age (± SD) was 5.95 (± 3.23) with range from 6 months to 13 years. Patients were complaining of delayed milestones, loss of acquired milestones, learning disabilities, convulsions, limb weakness, inability to walk and skeletal problems. All patients that have suffered from neurological manifestations not due to peroxisomal disorders were excluded. The study also included fourteen healthy age and sex matched subjects served as control group.

- Ethical consideration: Approval was obtained from pediatric department council of Cairo University. Written consent was taken from parents of the included children. The study was approved by Cairo University research ethics committee and National Research Centre ethics committee. All procedures, included individual data were treated with confidentiality following Helsinki Declaration.
- According to the classification of Wanders and Waterham, [7], subjects with peroxisomal disorders were divided into two groups:
  1. The PBD group (17 patients), which included one patient with ZS, 14 patients with NALD and two patients with RCDP;
  2. The single peroxisomal enzyme deficiency group (3 patients), which included two patients with XALD and one patient with adult Refsum disease.
- All patients were subjected to full history taking including three-generation pedigree analysis concerning parental consanguinity and similarly affected family members. Also, full clinical and neurological examination was done for each patient to detect any malformations or anomalies that usually co-exist with peroxisomal disorders. Developmental examination including both motor and intellectual skills was also done for each patient. Brain Magnetic Resonance Imaging (MRI) was done for each patient. Other Investigations were done according to the presentation of each patient e.g. visual evoked potential, fundus examination, Auditory brainstem response test (ABR) and hearing test.
- To verify the diagnosis, quantification of plasma VLCFAs (C26:0, C26:0/C22:0 and C24:0/C22:0) and phytanic acid was done using gas chromatography-mass spectrometry as described by Takemoto and his colleagues [11].

Methods

- Estimation of oxidative stress parameters was done by assessment of:
  a. Malondialdehyde (MDA) as a marker of lipid peroxidation using high performance liquid chromatography [12].
  b. Nitrites and nitrates as NO metabolites (NOx) that indicate endogenous NO formation using Griess reaction (Bioxytech Nitric Oxide Non-Enzymatic Assay) [13].
  c. SOD as an antioxidant using an immunosorbent technique kit [14].
- Estimation of freeoxide radicals was done in whole blood using Electron Spinning Resonance (ESR) instrument (Bruner Elexsys ES001 with microwave bridge 9GH2 (XN bone), Pruker Elexsys E500. The method is based on the spin trapping technique in combination with ESR measurement [15]. Blood was withdrawn carefully to keep red cells intact to avoid release of antioxidant enzymes into the blood plasma. Samples were collected with the spin trap being present in the syringes to make sure that the radicals are trapped immediately during the collection, kept on ice, incubated for a certain time and the ESR spectra were recorded on same day. We used a mixture of hydroxyl amines (TEMPONE-H/ TEMPONE) as a spin trapping solution where the hydroxyl radicals preferably react with TEMPONE by oxidizing it to an oxo-ammonium compound. A mixture of TEMPONE-H/TEMPONE +DMPO (5, 5-Dimethyl-1-pyrroline-N-oxide) is used as DMPO is a very effective scavenger of hydroxyl radicals generating methyl or hydroxymethyl radicals. As hydroxyl radicals oxidize TEMPONE and methyl radicals generate TEMPONE by oxidizing TEMPONE-H, these re-activities can be used to detect and quantify hydroxyl radicals.

STATISTICAL ANALYSIS

Statistical analysis was done using non parametric analysis. We used statistical computer program SPSS version 16 for windows (SPSS Inc. Chicago). All reported p-value < 0.05 was considered significant [16].

RESULTS

Fifteen cases (75%) showed positive parental consanguinity and13 cases (65%) had similarly affected family members. As regarding MRI examination in NALD patients (14 patients), white matter demyelination was found in 11 patients (78.6%) while brain atrophy was seen in two patients and cerebellar atrophy in one case (7%). White myelin demyelination was found in patient with ZS, one patient with RCDP and patients with single enzyme deficiency. One patient with RCDP showed diffuse brain atrophy.

Results of VLCFAs including C26:00, C26:00/C22:00, C24:00/C22:00 and phytanic acid showed higher levels in all patient groups compared to control group [Table/Fig-1]. Very high significant difference was found between patients with NALD and control group regarding VLCFAs, phytanic acid, MDA, NOx, SOD and free oxide radicals levels [Table/Fig-2]. Results of MDA and NOx among all other patients showed higher levels than in control subjects [Table/Fig-1]. However, SOD levels were lower in all twenty patients compared with control subjects [Table/Fig-1].

NB: Non parametric student t-test could not be applied to ZS, RCDP, XALD and adult Refsum disease patients due to the small number of patients (n= 1, 2, 2 & 2 respectively).

parameter	Refsum (n=1)	NALD (n=14)	RCDP (n=2)	XALD (n=2)	ZS (n=1)	Control (n=14)
C26:0 ug/ml	2.5	2.8	3.2	4.9	2.2	0.23
C26:0/C22:0	3.3	2.3	2.1	2.49	1.7	0.09
C24:0/C22:0	4.3	2.4	2.5	2.46	2.2	0.33
Phytanic acid ug/ml	25.6	4.4	8.2	13.3	9.5	2.07
MDA nmol/ml	2.4	2.7	3.7	3.1	4.6	1.06
Nitrites/Nitrates nmol/ml	77.3	76.1	88.8	84.6	95.7	61.9
SOD U/gHb	911	855.1	90.5	859.5	97.9	1066
Free oxide radicals	111.5	162.7	236.3	46.7	141.9	39.9

[Table/Fig-1]: Mean levels of biochemical markers in patients groups and control subjects.
VLCFA=very long chain fatty acid, NALD= Neonatal Adrenoleukodystrophy, ZS= Zellweger syndrome, RCDP= rhizomelicchondrodysplasiapunctata, XALD= X-linked adrenal leukodystrophy, MDA= Malondialdehyde, SOD=Superoxide Dismutase.P* is significant if ≤ 0.05, P** is more significant if ≤ 0.01

DISCUSSION

Oxidative stress via free radicals has been shown to participate in the pathogenesis of peroxisomal disorders [17].

In our society, the effect of consanguinity in the occurrence of rare recessive disorders was confirmed by the positive consanguinity in 75% of cases and similar affected family members in 65% of cases. This study showed that NALD was the commonest disorder among the patients (70%), followed by XALD (10%), RCDP (10%), ZS (5%) and adult Refsum disease (5%). However, Engelen et al., reported that XALD is the most common peroxisomal disorder [18]. This contradiction could be attributed to the different locations of the studies.

Variable	NALD		Control		P-value
	Mean ±SD	Range	Mean ±SD	Range	
C26:00 ug/ml	2.89±1.1	1.21 - 4.69	0.237 ±0.10	0.11-0.44	0.000**
C26:00/C22:00	2.32 ±1.59	0.89 - 5.75	0.099 ±0..095	0.01 - 0.35	0.000**
C24:00/C22:00	2.42 ±1.98	0.29 - 6.35	0.339 ±0.33	0.02 – 0.89	0.002**
Phytanic acid ug/ml	4.46 ±3.18	0.31-11.56	2.07 ±2.19	0.02 - 8.5	0.062
MDA nmol/ml	2.7 ±0.86	1.8 - 4.2	1.036 ±0.37	0.5 – 1.8	0.000**
Nitrites/Nitrates nmol/ml	76.18 ±11.56	54 - 99.9	61.9 ±10.27	51 - 89.3	0.014*
SOD U/gHb	855.71 ±256.5	110 - 1200	1066 ±130.1	790 - 1236	0.011*
Free Oxide Radicals	162.7±98.05	49.12-344.8	39.9±47.0	4.95-191.5	0.002**

[Table/Fig-2]: Differences in levels of VLCFAs, phytanic acid and oxidative stress parameters levels between NALD patients and control subjects.
VLCFA=very long chain fatty acid, NALD= Neonatal Adrenoleukodystrophy, MDA=Malondialdehyde, SOD=Superoxide Dismutase. P* is significant if ≤ 0.05, P** is more significant if ≤ 0.01

Measurement of plasma VLCFAs levels is the most common initial screen test and increased levels of C26:0, C24:0/C22:0 and C26:0/C22:0 is consistent with disturbance in peroxisomal fatty acid metabolism [19]. This study showed highly significant elevation in VLCFAs levels and ratios in NALD group compared to control subjects while phytanic acid results in the same group of patients showed no statistical difference. However, the results of VLCFAs and phytanic acid in Refsum, RCDP, XALD and ZS patients were higher compared to control group. Elevated VLCFA with normal level of phytanic acid in some peroxisomal disorders was previously reported by Wanders et al., and Bams et al., [20,21], while other studies reported increased levels of phytanic acid in peroxisomal diseases [4,19].

Free radicals, products of lipid peroxidation and disturbance in antioxidant mechanisms are considered hallmarks in oxidative stress condition. In the present study, NALD patients showed significant elevated levels of free radicals compared to healthy subjects. Refsum, RCDP, XALD and ZS patients also showed higher levels of free radicals than controls. Gilg et al., and Petrillo et al., reported elevated levels of free radicals in peroxisomal disorders [22,23].

MDA is one of the products of lipid peroxidation. It is a sensitive and reliable marker that indirectly reflects the intensity of cellular injury and ROS production [24]. MDA elevation was considered one of the earliest quantitative disease markers [24]. We reported a highly significant elevation level of MDA in NALD group compared with controls. Similarly, Refsum, RCDP, XALD and ZS patients showed higher levels of MDA than controls. This is in agreement with several studies that reported high levels of MDA in different groups of patients with peroxisomal disorders [25,26].

NO is the source of peroxynitrite which is considered one of RNS and plays major role in free radical mediated tissue damage [27]. Nitrites and nitrates were considered as indicators of NO formation [13]. In the present study, NOx showed significant elevation in NALD group compared to control subjects. Similarly, Refsum, RCDP, XALD and ZS patients showed higher levels of NOx than control group. Similar results were previously reported by Surdacki et al., who stated that NO production was highly elevated in infants diagnosed with ZS [28]. Dhaunsi et al., also reported that XALD patients had higher significant NO levels than control subjects [25].

Results of MDA and NO are coincident with many studies that reported increase oxidative stress parameters in peroxisomal disorders [4, 29].

We reported lower significant levels of SOD in NALD group compared with control group. Similarly, RCDP, XALD and Refsum patients showed lower levels of SOD than controls and the ZW patient had the lowest level. These results coincide with other studies that reported decreased levels of SOD in patients with peroxisomal disorders [4,19,26]. On the other hand, Petrillo et al reported similar SOD activity in peroxisomal disorder patients and control group [23], while Baarine et al., reported higher SOD in patients with peroxisomal disorders [10]. Over expression of SOD was also reported in oxidative stress conditions associated with neural disorders [30]. This confliction between all these different studies may be underscored by the fact that levels of SOD in peroxisomal disorders depend on the degree of disease severity, activity of the disease and exhaustion or depletion of the enzyme.

Central nervous system is considered of one of the primary targets for oxidative stress damage [27]. This explains the central and cortical demyelination in brain MRI in most patients included in the study. This also coincides with the findings of other studies [4,19]. Although we reported an increase in VLCFA levels in studied patients than controls, we cannot formally conclude if this accumulation is the direct cause for brain demylination or the elevated basal levels of ROS is the cause. Interestingly, it was found that the animal models of XALD do not express a clear disease phenotype and do not show evidence of oxidative stress in nervous tissue although they accumulate VLCFA in their central nervous system [26]. López et al., have found evidences for an early oxidative stress, occurring at presymptomatic stages in peroxisomal disorders [29]. The previous results suggest that oxidative imbalance in these patients may play a role in the disease pathogenesis and progression.

LIMITATION

The major limitation of this study is low number of patients. Correlations between levels of free radicals and degree of brain damage and the outcome of patients were better to be done.

CONCLUSION

Our results strongly support a role of free radicals in the pathophysiology of peroxisomal disorder patients and strengthen the importance of oxidative stress phenomenon in disease pathogenesis. Strategic plan should be directed to decrease accumulation of VLCFA, reduce ROS production and increase anti-oxidant enzymes concentration and activity.

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