



Original Article

Peroxidative degradation of proteins and lipids in undifferentiated connective tissue dysplasia in children, Ukraine

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Abstract

The aim of the study was to assess the state of lipid and protein peroxidation in children with undifferentiated connective tissue dysplasia (UCTD). In this study, 63 children (33 children with UCTD and 30 children without UCTD) were recruited and the indicators of lipid and protein peroxidation were measured. The enzymatic, colorimetric method was used to measure the level of total cholesterol (TC). The phospholipids were analyzed by liquid chromatography/mass spectrometry using electrospray ionization. Lipid peroxidation was studied by assessing the change of index and the end product of lipid peroxidation, malondialdehyde (MDA), using spectrophotometric method. Protein peroxidation - by the content of carbonylated protein based on 2,4 dinitrophenylhydrazones derivatization. The activity of the antioxidant defense system enzymes was assessed by measuring catalase (CAT) and superoxide dismutase (SOD). Our study revealed a significant increase of PPO products in the venous blood plasma and LPO products in erythrocytes in children with UCTD. Furthermore, an imbalance of the antioxidant defense system was observed in both blood plasma and erythrocytes membrane.

Keywords: UCTD, undifferentiated connective tissue dysplasia, protein peroxidation, lipid peroxidation, antioxidant enzymes

Introduction

Oxidative stress occurs in children with undifferentiated connective tissue dysplasia (UCTD). In the state of oxidative stress, peroxynitrite is overly synthesized during the interaction of nitric oxide (NO) and superoxide anion. In the case of non-radical decomposition of peroxynitrite, nitrogen nitrate (NO_3^-) is formed, and in the case of radical decomposition, an OH^- radical is generated (known as an activator of arginase) [1].

Oxidative stress leads, on the one hand, to an increase in the synthesis of collagen precursors, and on the other hand, to the limitation of the NO formation via endothelial NO synthase (eNOS) due to the competition of eNOS and arginase for a common substrate, L-arginine. The presence of such competition limits the availability of NO with the development of endothelial dysfunction and supports proinflammatory, prothrombotic, proliferative, and vasoconstrictive processes in the bodies of children with UCTD [2].



The impairment of the bioavailability of NO may also be associated with the production of a superoxide anion, which rapidly binds and inactivates NO. It was found that the production of superoxide by the vascular wall is increased in hypercholesterolemia, diabetes mellitus, arterial hypertension, smoking, and other processes [3-8]. The formation of highly toxic peroxynitrite (ONOO⁻), concomitant to the interaction of superoxide anion and NO, promotes the activation of lipid peroxidation (LPO) which results in the formation of aldehydes (including malondialdehyde), ketones, diene conjugates, Schiff's bases, and so on. In conditions of persistent violation of physiological equilibrium between anti- and pro-oxidant processes towards pro-oxidant, POL activation is possible, which is accompanied by damage to body cells [9]. Around 95% of oxygen is reduced to water in the mitochondria during oxidative phosphorylation, where the remaining 5% are converted into reactive oxygen species (ROS) through various reactions (usually enzymatic).

In addition to activating LPO, ROS might cause protein peroxidation (PPO) or oxidative modification of proteins, resulting in oxidative destruction of proteins in cells and tissues and deepening of membrane damage [10]. It should be noted that in a state of oxidative stress, ROS primarily affects proteins of plasma membranes, not lipids. The neutralization of cellular ROS relies on an antioxidant defense system (AODS) that combines several enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase, as well as some low molecular weight antioxidants - vitamin C, glutathione, and uric acid.

An analysis of modern scientific literature concludes that a significant number of works are devoted to LPO, while less attention is paid to the oxidative destruction of cell and tissue proteins. This is the novelty of this study by including oxidative protein degradation in the assessment. Protein modification has been suggested to play a role in signaling the change of cellular metabolism [11]. It is due to the fact that sulfo- and amino-hydroxyl groups of amino acids are subject to peroxidation, which leads to the crosslinking formation between proteins or between a protein and other NH₂-containing molecules.

Based on the foregoing, the general aim of the work was to study the state of oxidative modification of proteins and lipids, as well as the activity of SOD and CAT in children with UCTD. Therefore, the index of lipoproteins peroxide modification and end product of LPO process – malondialdehyde (MDA) were used as the indicator of LPO intensity. Free radicals generate the lipid peroxidation process in an organism. An increase in free radicals causes overproduction of MDA, which is the final products of polyunsaturated fatty acids peroxidation in the cells. MDA level is commonly known as a marker of oxidative stress [12]. Although others biomarkers can be used to measure the lipid peroxidation, the MDA quantification in pathologies (cancer, diabetes, Alzheimer's disease) and toxicology is the most utilized [13]. In addition, free radicals-induced protein oxidation was assessed through the content profile of carbonylated proteins. As for the AODS, the investigation was conducted based on the content of CAT and SOD in membrane structures and blood plasma, as suggested previously [14-17].

Methods

In this study, 63 children (28 girls and 35 boys) aged 11-18 years were included. Among them, 33 children (13 girls and 20 boys) with UCTD and 30 children (15 girls and 15 boys) without UCTD. The study was conducted under the Department of Pediatrics №1 Bogomolets National Medical University, Ukraine, between 2004-2019. The children were examined at the Children's Clinical Hospital №4, the clinical base of the Department of Pediatrics №1 Bogomolets National Medical University in Kyiv, during free respiratory and other pathologies or one month after an acute process. All children attended general education schools. The UCTD was diagnosed using a patented special table of phenotypic signs of UCTD [18]. Diagnosis of UCTD was decided when there were six or more phenotypic signs of dysplasia observed. Several examinations were conducted to establish the diagnosis as well as to determine other clinical and pathological features of both groups, including electrocardiographic and echocardiologic, abdominal ultrasound, esophagogastroduodenoscopy, histological examination of the gastric mucosa, and breath test for *Helicobacter pylori*.

The venous blood from each child was collected during the morning time on an empty stomach. A spectrophotometric method was then used to obtain the index of peroxide modification of lipoproteins, malondialdehyde (MDA) (collected from blood plasma and erythrocyte membranes). MDA was determined by using thiobarbituric acid (reagents are 0.025 M Tris-HCL buffer (pH 7.4) containing 0.175 M potassium chloride; 17% solution of trichloroacetic acid; 0.8% solution of 2-thiobarbituric acid) [19,20].

Carbonylated proteins were determined based on 2,4-dinitrophenylhydrazones (DNPH) derivatization [21]. A sample (1-1.5 mg protein/mL) was divided into two 1-mL portions; labeled as “Test” and “Blank”. The samples were precipitated with 10% trichloroacetic acid (final concentration). The samples were treated with an equal volume of 0.2% DNPH in HCl 2 M, meanwhile the Blank portions were only added with HCl 2 M. The samples were incubated at room temperature in the dark for 1 h, vortexing every 10 min, after that they were centrifuged for 15-20 minutes at 3000 g. The sediments were subsequently washed 3 times with ethanol-ethyl acetate (1:1, by volume). The pellets were carefully drained and dissolved in 3 mL carbamide 8 M, and then the samples were incubated for 5 minutes at 100°C. Insoluble debris was removed by centrifugation at 6000 g and 4°C. The reactive carbonyl content was calculated from its absorption peak (365 nm) using a molar absorption coefficient of 21.0 mM⁻¹cm⁻¹. The concentration of total protein was measured by Bradford method [22].

The determination of CAT activity was measured according to the Beutler method [14]. Catalase catalyzes the breakdown of H₂O₂ to H₂O and O₂. The rate of H₂O₂ decomposition by catalase was measured spectrometrically at 230 nm (optimum wavelength). Ethanol was added to stabilize the hemolysate by breaking down ‘complex II’ of catalase and H₂O₂. After the addition of 50 µL tris buffer, 900 µL H₂O₂ and 30 µL H₂O to the cuvettes, the system was incubated at 37°C for 10 minutes, the hemolysate was added, and, in the following 10 minutes, the decrease of optical density was measured against blank at 412 nm [15].

SOD activity was measured according to the method described by Fridovich [16]. This method employs xanthine and xanthine oxidase to generate superoxide radicals that react with p-iodonitrotetrazolium violet (INT) to form a red formazan dye that was measured at 505 nm. The assay medium consisted of the 0.01 mol/L phosphate buffer, a 3-cyclohexylamino-1-propanesulfonic acid (CAPS) buffer solution (50 mmol/L CAPS, 0.94 mmol/L EDTA, saturated NaOH (pH 10.2), solution of substrate (0.05 mmol/L xanthine, 0.025 mmol/L INT), and 80 U/L xanthine oxidase [17].

The cholesterol was determined using the enzymatic, colorimetric method. Cholesterol in the sample was first hydrolyzed into free cholesterol and fatty acid using enzyme cholesterol esterase. The free cholesterol was then oxidized by cholesterol oxidase to give cholestene-3-one and hydrogen peroxide. The peroxide then reacted with 4- aminoantipyrine (AP) and hydroxybenzoate, catalyzed by peroxidase, to form a red compound. The color intensity as measured at 505 nm was proportional to the total cholesterol present in the sample.

The phospholipids were analyzed by liquid chromatography/mass spectrometry using electrospray ionization [23].

The data were statistically analyzed using Microsoft Excel 07 and Statistica 5.0, where differences between the compared values were considered significant at p<0.05. When analyzing variation series that differ in shape from the normal distribution, nonparametric criteria were used.

Results

Among children with signs of UCTD, the MASS phenotype was observed in 21 (63.6%) children, Ehlers-like phenotype in 5 children, Marfan-like phenotype in 7 (21.2%) (**Table 1**). MASS phenotype has clinical features that included mitral valve prolapse, aortic root dilation, skin striae, and skeletal features include curvature of the spine (scoliosis), chest wall deformities, and joint hypermobility. According to the data of height, chest circumference, and body weight, most of the children lagged in physical development by two stigma deviations from the normal age. The asthenic constitution was observed in 30 (90.9%) children with UCTD, according to the height-mass index (BMI), which was <18.5 in this group of children. The remaining 3 (9.1%)

children with UCTD had BMI within the normal range. In children without signs of UCTD, a lag in physical development was observed in 3 children (10%). The majority of UCTD cases (27, 81.8%) complained of the manifestations of chronic nonspecific intoxication such as increased fatigue, emotional lability, restless sleep, excessive sweating, headache in the afternoon, arthralgia, and myalgia, while only 4 (13.3%) of those without UCTD had chronic nonspecific intoxication symptoms (**Table 1**).

Blood pressure was below the age norm in 29 (87.9%) children with signs of UCTD and 6 (20%) children without UCTD. To a certain extent, the lowered blood pressure can explain the muffledness of the heart sounds, which are caused by the general hypotonia of the body and, in particular, by the weakness of the heart muscle. Examination of the lymphatic system of children revealed an increase in regional lymph nodes (posterior cervical, submandibular, axillary, and inguinal) in the form of micropoliadenia, which was observed in 29 (87.9%) children with UCTD and 6 (20%) children without UCTD. Chronic tonsillitis was diagnosed in 29 (87.9%) cases and 7 (23.3%) children without CTD (**Table 1**).

Changes in the heart, which were manifested by functional murmurs at the apex of the heart and the V point (Erb's point) and some muffledness of heart sounds, were recorded in 29 (87.9%) children with UCTD and 7 (23.3%) children without UCTD. Children with UCTD had rhythm disturbances in the form of sinus bradyarrhythmia, changes in conduction - incomplete bundle branch blocks, and ST interval inversion. We also observed autonomic dysfunction of the cardiovascular system in 30 (91%) children with UCTD. However, clinical examination of the children revealed no pathological changes in the lungs.

The palpation of the abdomen revealed an increase in the size of the liver among 20 (64.5%) UCTD cases, which on average protruded on 1-2 cm from under the edge of the costal arch along l. medioclavicularis dextra. Palpation in these children revealed a soft, rounded, painless edge of the liver. Symptoms of Ortner, Murphy, Kera-Gausman, Egorov, and others were detected in almost all children. Abdominal ultrasound of children from the UCTD group revealed 100% dysfunction of the bile tract (DBT), which was combined with an anomaly in the gallbladder. In children of the control group, DBT was found only in 10% of cases (**Table 1**). Eleven (32.3%) UCTD children who complained of abdominal pain related to food intake, nausea, decreased appetite were diagnosed with chronic gastritis and *H. pylori* positive. It was confirmed by the results of esophagogastroduodenoscopy, histological examination of the gastric mucosa, and breath test for *H. pylori*. Children without UCTD had no clinical signs of chronic gastritis.

Table 1. Clinical characteristics of children with and without UCTD

Clinical characteristics	Children with UCTD n=33		Control group n=30	
	n	%	n	%
MASS phenotype	21	63.6	0	0.0
Ehlers-like phenotype	5	15.2	0	0.0
Marfan-like phenotype	7	21.2	0	0.0
Constitutional type (asthenic physique)	30	90.9	0	0.0
Manifestations of chronic nonspecific intoxication	27	81.8	4	13.3
Arterial hypotension	29	87.9	6	20.0
Lymphadenopathy manifestations	29	87.9	6	20.0
Manifestations of chronic tonsillitis, pharyngitis	29	87.9	7	23.3
Heart auscultation changes	29	87.9	7	23.3
Cardiac type autonomic dysfunction	30	91	7	23.3
Development abnormalities of the gallbladder	33	100	3	10.0
Biliary tract dysfunction with enlarged liver	20	64.5	2	6.7
Chronic gastritis, <i>H. pylori</i> -positive	11	32.3	0	0.0
Violation of the functional state of the intestine and the phenomena of intestinal dysbiosis	25	74.2	3	10.0

The obtained PPO and LPO indices, as well as the activity of antioxidant enzymes (SOD and CAT) in the venous blood plasma of the examined children, are presented in **Table 2**. There was a significant increase in PPO products (2.61 ± 0.08 CU/mL) and an index of lipoprotein peroxide modification (1.30 ± 0.04 conventional unit (CU)/mL) in the blood plasma of children with UCTD compared to controls (1.90 ± 0.03 CU/mL; $p < 0.05$ and 1.10 ± 0.03 CU/mL, respectively; $p < 0.05$).

In children with UCTD, there was also a significant increase in the level of plasma carbonylated proteins (0.63 ± 0.02 CU/mL) compared to children without UCTD (0.51 ± 0.02 CU/mL; $p < 0.05$).

Table 2. Indicators of protein and lipid peroxidation in plasma of children with and without UCTD

Indicators of blood plasma	Group		p-value
	Children with UCTD Mean \pm SD n=33	Control group Mean \pm SD n=30	
Products of free radical oxidation of proteins (CU/mL), mean \pm SD	2.61 ± 0.08	1.90 ± 0.03	< 0.05
Carbonylated proteins in LDL and VLDL apoproteins, CU/mL	0.63 ± 0.02	0.51 ± 0.02	< 0.05
Index of peroxide modification of lipoproteins	1.30 ± 0.04	1.10 ± 0.03	< 0.05
MDA, kmol/min.mg	0.40 ± 0.05	0.30 ± 0.07	> 0.05
SOD, $\mu\text{mol}/\text{min. mg}$ of protein	2.60 ± 0.20	1.60 ± 0.09	< 0.05
CAT, mkat/L	23.90 ± 1.8	29.20 ± 1.90	< 0.05

A study of the lipid spectrum of the erythrocyte membrane (total cholesterol-TL and phospholipid levels - PL) was carried out to confirm the peroxidation-induced membrane damage. The investigation also included the analysis of MDA and CAT indicators in the membrane of erythrocytes. The indicators in adolescent children with UCTD were compared with those without dysplasia (**Table 3**).

Table 3. Lipid spectrum, MDA, CAT collected from erythrocyte membrane of children with and without UCTD

Indicators in the erythrocyte membrane	Group		p-value
	Children with UCTD Mean \pm SD n=33	Control group Mean \pm SD n=30	
Total cholesterol (TC), $\mu\text{mol}/\text{mg}$	$0.35 \pm 0.01^*$	0.30 ± 0.01	< 0.05
Phospholipids, $\mu\text{mol mg}$	0.27 ± 0.03	0.29 ± 0.03	> 0.05
MDA, $\mu\text{mol}/\text{min. mg}$ of protein	$1.05 \pm 0.07^*$	0.75 ± 0.03	< 0.05
CAT, CU/mg	$1.88 \pm 0.16^*$	2.80 ± 0.80	< 0.05

Discussion

The protection of cells from the reduction, induced by one-electron oxygen intermediates, relies heavily on enzymatic AODS, in which SOD is the key component. This enzyme neutralizes superoxide anion radical formed during the enzymatic one-electron reduction of the oxygen molecule ($\text{O}_2 + \text{E} \rightarrow \text{O}_2^-$) by converting it into less reactive molecules; hydrogen peroxide - H_2O_2 and triplet oxygen ($\text{O}_2^- + \text{O}_2^- \rightarrow \text{O}_2 + \text{H}_2\text{O}_2$). Subsequently, H_2O_2 is decomposed by catalase as well as peroxidases with various substrate specificities.

Previous studies have shown that there is an increase in the levels of the initial LPO products - lipid hydroperoxides, and the final products - MDA, with a simultaneous decrease in AODS indicators (reduced glutathione, glutathione peroxidase, G6PD) in the erythrocytes of venous blood of 7-12 years old children with UCTD [24]. Similar results were obtained in our present study, where UCTD children within the puberty age experienced a significant increase in the activity of SOD plasma (2.60 ± 0.20 $\mu\text{mol}/\text{min. mg}$ of protein) compared to those without UCTD. A significant decrease in the activity of CAT was also observed in children with UCTD compared to the control group.

Our data suggested that the proteins of venous blood plasma were more sensitive to peroxidation processes compared to lipids in UCTD children. This might be why levels of MDA (end product of LPO) observed between the studied group were not significant.

The data obtained in this study could indicate impairment of the AODS adolescent children suffering from UCTD. The increase in SOD activity is possibly associated with a compensatory reaction of the body of a pubertal child to the formation and circulation of a large amount of superoxide radicals in the blood plasma, which thereby protects the vessels from the effects of

highly active oxygen metabolites, converting it into hydroperoxides. At the same time, a decrease in CAT activity in pubertal children with UCTD could lead to the accumulation of aggressive hydroperoxides in blood plasma with destructive activity aimed at blood cells. According to the results presented in **Table 3**, there was an impairment of the lipid layer structure in the membranes of erythrocytes in children with UCTD; the cholesterol content increased significantly with a normal level of total phospholipids ($p > 0.05$) [25-28].

It should be noted that membrane lipids are distinguished by a variety of structural forms, forming complexes with proteins, and can affect the conformation and biological properties of the latter in different ways, damaging the cellular function and interaction. Nonetheless, activation of LPO and decreasing AODS activity are possibly required as one of the mechanisms of erythrocyte membrane reconstruction.

Different from the profile of the venous blood plasma, MDA was observed significantly higher in the erythrocyte membranes collected from the UCTD patients compared to that of the control group. Moreover, there was a significant decrease in the antioxidant defense system - CAT indicator in the membranes of venous erythrocytes in UCTD children compared with those in the control group. Taken altogether, these suggest the occurrence of LPO in erythrocytes with AODS impairment. Thus, an increase of PPO products in blood plasma and LPO in erythrocytes along with impaired AODS (proven by the increase in SOD activity and decrease in CAT activity) could be alarming indicators. Our study suggests that excessive peroxidation of proteins and lipids could lead to cellular destruction and the development of pathological processes in various organs and systems of the child's body, where collagen breakdown might increase.

Conclusions

There is an impairment in the system of peroxidation of plasma proteins of venous blood in adolescent children with UCTD, evidenced by the changes of carbonyl group contents in apoproteins of low-density lipoproteins and very low-density lipoproteins, the index of peroxide modification of lipoproteins. An increase in TC suggested the occurrence of LPO in erythrocyte membranes of the UCTD patients. There is an imbalance of antioxidant enzymes (superoxide dismutase and catalase) in adolescent children with UCTD. More studies need to be carried out to examine the correlation of the aforementioned indicators with the severity of UCTD.

Declarations

Ethics approval

The protocol used in this study was approved by the Commission on Bioethical Expertise and Ethics of Scientific Research of Bogomolets National Medical University, Kyiv, Ukraine. Informed consent was obtained from all participants prior to their participation in the study.

Conflict of interest

The authors declare that there is no conflict of interest.

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