LIPID PEROXIDATION AND OXIDATIVE PROTEIN PRODUCTS IN CHILDREN WITH EPISODIC FEVER OF UNKNOWN ORIGIN

LIPIDNA PEROKSIDACIJA I OKSIDATIVNI PROTEINSKI PRODUKTI KOD DECE SA EPIZODIČNOM GROZNICOM NEPOZNATOG UZROKA

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Summary

Background: Episodic fever syndromes are commonly seen in pediatric practice. Episodic fever of unknown origin (FUO) lasts for a few days or weeks and is followed by a fever-free period with a sense of well-being. In this condition, activated neutrophils and monocytes intensively generate reactive oxidative species that may further damage various molecules. The aim of the study was to evaluate oxidative stress biomarkers, lipid peroxidation in erythrocytes and plasma, and advanced oxidation protein products (AOPP) in children with episodic FUO.

Methods: The study enrolled 25 children with episodic FUO in afebrile phase and 25 healthy children as controls. Lipid peroxidation was evaluated by measuring malondialdehyde (MDA) production with the thiobarbituric-acid-reactive substances (TBARS) assay in erythrocytes and plasma. Oxidative modification of proteins was measured spectrophotometrically by the determination of AOPP in plasma.

Results: Mean duration of episodic fevers was 3.96±2.8 years. Erythrocyte MDA levels were higher in children with FUO than in controls (86.26±10.75 vs. 78.0±3.21 nmol/g hemoglobin), although not significantly (p=0.202). The MDA plasma concentrations were similar (2.42±0.35 vs. 2.41±0.39 μmol/L) between the groups (p=0.732). Unexpectedly, levels of AOPP were significantly lower in children with FUO than in healthy controls (18.8±5.04 vs. 25.1±3.35 μmol/L, p=0.047).

Kratak sadržaj

Uvod: Sindromi epizodične ili rekurentne groznice često se sreću u pedijatrijskoj praksi. Epizodična groznica nepoznatog uzroka (FUO) traje nekoliko dana do nekoliko nedelja, nakon čega sledi miran period bez povisene temperature uz osećaj potpunog zdravlja. U ovim stanjima, aktivirani neutrofili i monociti intenzivno produkuju reaktivne kiseonične vrste koje naknadno mogu oštetiti različite molekule. Cilj našeg rada bio je oceniti biomarkere oksidativnog stresa, odnosno lipidnu peroksidaciju u eritrocitima i plazmi, kao i uznapredovale oksidativne proteinske produkte (AOPP) kod dece sa epizodičnom FUO.


Rezultati: Srednje vreme trajanja epizodičnih groznica bilo je 3.96±2.8 godina. Vrednosti MDA u eritrocitima su bile više kod dece sa epizodičnom FUO nego kod zdrave dece (86.26±10.75 vs. 78.0±3.21 nmol/g hemoglobina), iako ne statistički značajno (p=0.202). Koncentracije MDA u plazmi su bile slične kod ove dve grupe dece (2.42±0.35 vs. 2.41±0.39 μmol/L, p=0.732). Nečekivano, nivoi AOPP-a su značajno bili manji kod dece sa FUO nego kod zdravih kontrolnih subjekata (18.8±5.04 vs. 25.1±3.35 μmol/L, p=0.047).

List of non-standard abbreviations: AID, autoinflammatory diseases; AOPP, advanced oxidation protein products; FUO, fever of unknown origin; MDA, malondialdehyde; TBARS, thiobarbituric-acid-reactive substances.
Conclusions: Episodic fevers of unknown origin with an average duration of 3.96±2.8 years do not cause significant oxidative modifications of lipids and proteins in children.

Keywords: blood proteins, fever of unknown origin, malondialdehyde, pediatrics, reactive oxygen species

Introduction

Fever is one of the most common signs in pediatric practice. Children, especially in the first years of life, have about 10 self-limited viral illnesses accompanied with fever. Sometimes the origin of fever remains unknown, and if the diagnosis is uncertain after 1 week of intensive evaluation, the fever is designated as a fever of unknown origin (FUO). FUO may occur as a single illness, where fever ≥38.0 °C lasts at least 3 weeks. On the other hand, recurrent or episodic FUOs are defined as three or more fevers ≥38.0 °C, that last for a few days to a few weeks, with a fever-free period and a sense of well-being (1–3). These syndromes represent a diagnostic challenge and usually the right diagnosis is delayed. According to the results of various studies, the main causes of FUO are infections. However, there are numerous noninfectious FUO. The second most frequent single FUOs are of inflammatory and malignant origin or due to collagen vascular disease (2, 4, 5). Beside infections, other causes of episodic FUO are more commonly hereditary, autoinflammatory and sometimes autoimmune diseases (2–4).

The most prevalent reasons of recurrent or episodic fever syndromes in pediatric practice are autoinflammatory diseases (AID). This is a group of hereditary diseases, monogenic or multifactorial (Table I), characterized by seemingly unprovoked episodes of fever and localized inflammation (pyelonephritis or pleuritis, synovitis, skin rash, fatigue, etc.) (1, 2, 5). Most of the AID are caused by mutations in the genes coding inflammasome sequences or its regulatory molecules in the cells of the innate immune system. A defect in the inflammasome control leads to interleukin-1 (IL-1) pathway disorders, without involvement of a distinct pathogen or the adaptive immune system. The caspase-1 inflammasome dysregulation results in diminished control over the IL-1 secretion through the inflammasome precursor processing and increased secretion of this proinflammatory cytokine (1, 6).

In chronic episodic FUO there is a cyclic activation of leukocytes, mostly neutrophils, monocytes, macrophages and dendritic cells, that now intensively secrete lymphokines and endogenous pyrogenic cytokines, such as IL-1β, IL-6, TNF-alpha (7). Interleukin-1β plays an important part in the inflammatory response. It resets the hypothalamic thermoregulatory center causing fever, induces leukocytosis, cachexia, hyperalgnesia, increases acute-phase protein synthesis, etc. Beside cytokines, activated neutrophils and monocytes produce significant amounts of reactive oxygen species (ROS) and reactive nitrogen intermediates via the NADPH oxidase complex and myeloperoxidase system during phagocyte oxidative burst (8). In these instances, reactive oxygen/nitrogen species may cause cell injury via the membrane lipid peroxidation and oxidative modification of carbohydrates, proteins and nucleic acids.

A positive feedback loop between IL-1β secretion, phagocyte activation and ROS production may aggravate inflammation and oxidative stress. Interleukin-1β induces neutrophilia and activation of phagocytic NADPH oxidase (6, 9, 10). Activated phagocytes in turn produce the proinflammatory cytokines (IL-1, TNF-α, and IL-6) (8), while ROS regulate IL-1β production through the inflammasome and nuclear factor-κ B activation, and recruitment of phagocytes into the affected tissues (11). Because of
The role of oxidative stress in inflammation has been described in the pathogenesis of many diseases, including the autoinflammatory (8, 9, 12). In some patients with AID subclinical inflammation and oxidative stress have been reported during fever-free periods (13–15). Considering all the abovementioned, there is perhaps enhanced oxidant production and accumulation of oxidative stress damage in persons with episodic FUO.

The goal of our study was to determine oxidative stress biomarkers in children with episodic fever of unknown origin, through the assessment of lipid peroxidation, both in erythrocytes and plasma, as well as advanced oxidation protein products. Our intention was to generally assess oxidative stress damage in these persons, when they are not directly affected by a febrile phase.

**Patients and Methods**

The study was conducted in compliance with the Declaration of Helsinki and Good Clinical Practice (GCP) Guidelines. The relevant study documents have been approved by an Independent Ethics Committee. The patients’ informed consent forms were provided in both written and oral form and signed by parents and, when possible, the patients.

The study group comprised 25 eligible children with episodic FUO referred to the Department of Pediatric Rheumatology, Clinical Center in Niš, Serbia. There were 14 (56%) boys and 11 (44%) girls with episodic FUO, with febrile episodes recurring for at least one year, without a febrile episode in the previous two weeks. During the thorough diagnostic procedure, infectious, autoimmune, metabolic and potentially malignant diseases were excluded. The children were not on drug therapy in the afebrile period when the blood samples were collected. A total of 25 healthy children who accepted to donate blood samples, taken for regular health checkups, comprised the control group. They were age and sex-matched to the children with episodic FUO.

We collected the whole blood samples with EDTA, after which plasma and washed erythrocytes were separated by a centrifuge. Lipid peroxidation (LP) was evaluated by measuring malondialdehyde (MDA) production with the thiobarbituric acid reactive substances (TBARS) assay in plasma and washed erythrocytes. Protein oxidation was measured by the determination of advanced oxidation protein products (AOPP) in plasma.

TBARS in erythrocytes were assessed spectrophotometrically according to the Jain et al. (16) method. Trichloroacetic acid and tertiary butyl alcohol were added to erythrocytes in phosphate buffer (pH 7.4) forming the chromogen. The absorption was measured at 532 nm wavelength. MDA concentration was expressed as nanomoles per gram of hemoglobin. TBARS concentration in plasma was determined spectrophotometrically according to the Andreewa et al. (17) method. The method is based on the reaction of MDA with thiobarbituric acid, at a high temperature and low pH. Measurement of MDA-TBA 2 chromogen is then assessed at 532 nm wavelength.

AOPP in plasma were determined spectrophotometrically according to chloramine T solution, which is then assessed at 340 nm (18).

Complete blood count, erythrocyte sedimentation rate, and C-reactive protein (CRP) and albumin concentrations were measured at the Biochemical Laboratory of the Clinic of Children’s Internal Disease in Niš, Serbia.

Complete blood count parameters (erythrocytes, leukocytes, platelets and hemoglobin) were assessed using the COULTER® AcT Diff Analyzer (Beckman Coulter Corporation, Hialeah, FL, USA), an automated hematology analyzer with a complete reagent system and authentic Coulter technology: triplicate counting, proven Coulter histogram differential, patented sweep flow technology and extended platelet counting.

For CRP and albumins concentration measurement, we used a fully automated Erba Mannheim XL600 analyzer with photometric tests and commercially available reagents of the manufacturer (ERBA Diagnostics Mannheim GmbH, Baden-Wurttemberg, Germany).

Erythrocyte sedimentation rate was assessed using the Westengard ESR method (19).

The results were expressed as mean ± standard deviation or median ± interquartile range values, as appropriate. Statistical analysis of biochemical parameters was conducted with the Mann-Whitney U-test or Students t-test, with statistical significance at p<0.05. For correlation between the parameters, we used Spearman’s rank order correlation. Statistical analysis was performed using the SPSS 17.0 (SPSS, Chicago, IL, USA) statistical program.

**Results**

Average age of the children with episodic FUO was 10.7±4.0 years, and 11.2±3.9 years that of the healthy children. Mean duration of a fever episode was 3.53±1.26 days, and the average duration of recurrent episodes was 3.96±2.8 years (range: 1–6.7).

The children with episodic FUO reported occasionally other symptoms, and among the most frequent were: abdominal pain (70%), lymphadenopathy (62.5%), headache (45.8%), polyarthralgia (33.3%),...
urticarial skin rash (33.3%), myalgia (29.2%), malaise (29.2%), oligoarthralgia (20.8%) and monoarthralgia (16.7%).

Levels of erythrocyte MDA were higher in children with episodic FUO than in healthy children, but without statistical significance (p=0.202). There was no difference in MDA concentrations in plasma (p=0.732) between the groups. Interestingly, levels of AOPP were significantly lower in children with episodic FUO than in controls (p=0.047). The levels of MDA and AOPP are shown in Table II.

Correlation between the examined oxidative parameters was low (0.1 to 0.4), except for MDA erythrocytes and AOPP levels in children with FUO that showed a moderate but statistically significant positive correlation (rs=0.516, p=0.02).

Also, erythrocyte MDA levels correlated positively with the albumin levels in the children with episodic FUO (rs=0.476, p=0.046). There were no other significant correlations between the oxidative stress biomarkers and C-reactive protein, albumins or leukocytes (Table III).

There was no significant difference in the complete blood count between the groups. Demographic data, complete blood count and inflammatory parameters are shown in Table III.

### Table II The results of MDA and AOPP levels in the study group and controls.

<table>
<thead>
<tr>
<th></th>
<th>MDA erythrocytes (nmol/g Hgb)</th>
<th>MDA plasma (μmol/L)</th>
<th>AOPP (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Children with FUO</td>
<td>86.26±10.75</td>
<td>2.42±0.35</td>
<td>18.8±5.04</td>
</tr>
<tr>
<td>Healthy children</td>
<td>78.0±3.21</td>
<td>2.41±0.39</td>
<td>25.1±3.35*</td>
</tr>
</tbody>
</table>

* statistically significant for p<0.05 compared to study group

AOPP – advanced oxidation protein products; Hgb – hemoglobin; MDA – malondialdehyde.

### Table III Demographic data, blood count and inflammatory parameters.

<table>
<thead>
<tr>
<th></th>
<th>Children with FUO (n=25)</th>
<th>Healthy children (n=25)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>10.7±4.0</td>
<td>11.2±3.9</td>
<td>–</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>16.7±2.1</td>
<td>17.2±3.3</td>
<td>–</td>
</tr>
<tr>
<td>RBC (×10¹²/L)</td>
<td>4.583±0.228</td>
<td>4.602±0.188</td>
<td>0.76</td>
</tr>
<tr>
<td>HGB (g/L)</td>
<td>133.80±14.4</td>
<td>135.50±10.17</td>
<td>0.35</td>
</tr>
<tr>
<td>WBC (×10⁹/L)</td>
<td>7.08±3.17</td>
<td>7.34±2.24</td>
<td>0.84</td>
</tr>
<tr>
<td>PLT (×10⁹/L)</td>
<td>352.43±80.6</td>
<td>291.2±57.5</td>
<td>0.51</td>
</tr>
<tr>
<td>ESR</td>
<td>9±8.8</td>
<td>9.1±9.9</td>
<td>0.98</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>1.93±2.78</td>
<td>0.68±1.9</td>
<td>0.27</td>
</tr>
<tr>
<td>Albumins (g/L)</td>
<td>47.28±3.75</td>
<td>45.18±5.04</td>
<td>0.22</td>
</tr>
</tbody>
</table>

CRP – C-reactive protein; ESR – erythrocyte sedimentation rate; PLT – thrombocyte count; RBC – erythrocyte count; WBC – leukocyte count.

Discussion

Fever is an important adaptive mechanism in the fight against infections. However, in the innate immune system disorders with spontaneous inflammasome activity, prolonged fever and inflammation may cause damage to the biomolecules and cells (7).

Malondialdehyde is one of the LP end-products and, since it is not generated exclusively through LP, it is considered as a general indicator of oxidative stress (8, 12). One of the often used and valid models for studying the effects of oxidative stress and lipid peroxidation are erythrocyte membranes (20).

Although the levels of erythrocyte MDA in our study were higher in the children with episodic FUO than in controls, the difference did not reach statistical significance. This result most probably reflects an effective erythrocytes’ antioxidative system response and sufficient protective concentrations of plasma antioxidants in these conditions (12, 21).

Plasma MDA originates from peroxidation of plasma lipids, thrombocytes, endothelial and other cells. Its level has been used as an indirect indicator of tissue LP, as lipid peroxidation products diffuse from damaged tissues and sites of inflammation into the circulation (12, 22, 23). Plasma MDA values were very similar between our groups, suggesting no significant tissue damage in children with episodic FUO. It is important to note that both erythrocyte and plasma MDA values were assessed in the afebrile phase, because lipid peroxidation products are often found in inflammatory diseases and show significantly higher values in the acute period of disease than in remission (12, 24, 25). The significant positive correlation of erythrocyte MDA with AOPP and albumins in our study may indicate elevated ROS levels in the febrile phase of episodic FUO and their modifying effects on lipids and proteins in this phase.

Albumins provide very important antioxidant defense activity in the plasma, especially their reduced thiol groups, that protect other macromolecules from oxidative injury (26). Advanced oxidation protein products are oxidative stress biomarkers in plasma, mostly derived from albumins, and to a lesser extent from fibrinogen and lipoproteins. They are normally formed in small amounts and their concen-
trations steadily increase with age (27, 28). AOPP are also recognized as markers of inflammation, with high levels being reported in diseases such as diabetes, chronic renal failure, hyperlipidemia, etc. (29, 30). Neutrophil oxidative potential was shown to be directly involved in plasma AOPP formation through the activity of myeloperoxidase, that is mostly abundant in these cells (8, 27, 28).

The higher values of AOPP in healthy children were unexpected, although the probability value was borderline significant (p=0.047). AOPP concentrations were not increased in the afebrile phase in the children with episodic FUO. It has been determined that AOPP levels are significantly higher in the attack period of inflammatory diseases than in remission and in controls (14, 28, 31). In the study of Keskin et al. (28), levels of AOPP were significantly higher in the active stage of Henoch–Schönlein purpura, a systemic inflammation of the blood vessels that commonly affects children, than in remission stage and in healthy controls. Also, the AOPP levels were similar in the remission stage and in controls.

We showed that episodic fevers that persist for approximately four years are not accompanied by significant AOPP accumulation, unlike in chronic inflammatory diseases. In the fever-free period, AOPP are normally cleared from plasma by the mononuclear phagocyte system (16, 27, 28). Also, free oxygen radicals, that are gradually and episodically released during the phases of inflammation, may increase the antioxidant defense capacity by the activation of enzymatic defense systems and better counteract ROS effects. This is opposite to prolonged or continuous inflammation in chronic diseases, where the total antioxidant capacity becomes reduced due to the high consumption (32, 33).

In conclusion, our study shows that episodic fevers with an average duration of four years do not cause significant oxidative modifications of lipids and proteins, and there is no substantial oxidative stress in children with these conditions. Among the examined parameters, erythrocyte membranes were the most vulnerable to ROS, and these oxidative changes endure the longest in the circulation. Decreased AOPP values in episodic FUO could be the result of the compensating, higher antioxidative capacity of the blood, induced by reactive molecular species.

Acknowledgments. The authors acknowledge the financial support within the Project III 41018: »Preventive, therapeutic and ethical approach in preclinical and clinical studies of the genesis and modulators of redox cell signaling in immune, inflammatory and proliferative cell response« of the Ministry of Education, Science and Technological Development of the Republic of Serbia.

Conflict of interest statement
The authors stated that there are no conflicts of interest regarding the publication of this article.

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Received: February 12, 2013
Accepted: May 26, 2013