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DNA damage and oxidative status in PFAPA syndrome

Selahattin Tuğrul [a], Remzi Doğan [b,*], Abdurrahim Kocyigit [c], Emel Torun [d], Erol Senturk [a], Orhan Ozturan [a]

[a] Bezmialem Vakif University, Faculty of Medicine, Department of Otorhinolaryngology, Fatih, Istanbul, Turkey
[b] Bayrampasa State Hospital, Department of Otorhinolaryngology, Bayrampasa, Istanbul, Turkey
[c] Bezmialem Vakif University, Faculty of Medicine, Department of Biochemistry, Fatih, Istanbul, Turkey
[d] Bezmialem Vakif University, Faculty of Medicine, Department of Paediatrics, Fatih, Istanbul, Turkey

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ABSTRACT

Objective: PFAPA syndrome is a clinical entity of unknown etiology which presents with periodic episodes of fever, aphthous stomatitis, tonsillitis or pharyngitis, and cervical adenitis. In this study we investigated DNA damage and the oxidative stress parameters in patients diagnosed with PFAPA, to elucidate the underlying pathophysiological mechanism of this syndrome.
Methods: Thirty-one patients diagnosed with PFAPA (Group 1), 22 patients diagnosed with normal tonsillitis or pharyngitis (Group 2), and 20 healthy volunteers (Group 3) were included in our study. Heparinized peripheral blood samples were drawn from all patients and volunteers. DNA damage was assessed by single cell alkaline electrophoresis assay in peripheral mononuclear leukocytes. Plasma levels of total antioxidant status (TAS) and total oxidative status (TOS) were determined by using a novel automated measurement method, and oxidative stress index (OSI) was calculated.
Results: DNA damage in the mononuclear leukocytes of Group 1 was significantly higher than that of Group 2 and Group 3. The oxidative stress parameters revealed that the TOS and OSI values of Group 1 were significantly higher than those of Group 2 and Group 3. TAS values of Group 1 were significantly lower than those of Group 2 and Group 3. Correlation analysis of Group 1 demonstrated a significant correlation between TOS, one of the oxidative stress parameters, and DNA damage. Correlations between DNA damage and C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) values were also significant.
Conclusion: Our study indicated that both the inflammatory and the oxidative stress parameters were significantly increased in patients with PFAPA syndrome, accompanied by a significant positive correlation between DNA damage and oxidative stress.

1. Introduction

PFAPA syndrome, first described by Marshall et al. [1] in 1987 as a syndrome affecting the pediatric age group, is a clinical entity of unknown etiology, which consists of recurrent periodic fever, aphthous stomatitis, tonsillitis or pharyngitis, and cervical lymphadenopathy [2].

Periodic fever usually occurs regularly every 3–8 weeks, and the febrile state generally continues for 3–6 days. There may also be some other accompanying symptoms, such as nausea, vomiting, headache, abdominal pain, muscle pains, diarrhea, arthralgia, cough, coryza and rash [3]. Diagnosis is reached by the presence of the above clinical criteria, plus by the exclusion of other periodic fever syndromes [4]. In current clinical practice, the diagnosis of PFAPA follows the criteria proposed by Thomas et al., and achieved widespread acceptance [2]. Viral and autoimmune mechanisms are being considered in the etiology of PFAPA, but so far the exact cause remains unidentified [1,5]. Studies on PFAPA syndrome revealed an increase in proinflammatory cytokines and a decrease in anti-inflammatory cytokines (during attacks) [6]. Increased levels of TNF-α, IFN-γ and IL-6 during attacks are also in favor of an ongoing inflammatory process [1,7]. Considering the above information, we have designed this study based on the thought that this inflammatory process would generate an oxidative stress in the organism.

Oxidative stress (OS) is an indicator of the disturbed balance between the generation of reactive oxygen species (ROS) and the antioxidant mechanisms set forth to detoxify those free radicals. In

* Corresponding author. Tel.: +90 505 7915844; fax: +90 212 544 35 94.
E-mail addresses: selahattintugrul@yahoo.com (S. Tuğrul), dr.remzidogan@gmail.com (R. Doğan), abdurrahimkocyigit@yahoo.com (A. Kocyigit), dr.emeltorun@gmail.com (E. Torun), erolsent@gmail.com (E. Senturk), orhanent@yahoo.com (O. Ozturan).

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normal physiological circumstances, ROS is not hazardous for the organism since it is balanced by antioxidants. When ROS concentrations exceed physiologic values, antioxidants like the enzymes superoxide dismutase (SOD), glutathione peroxidase (GSHPx), and catalase (CAT), and other non-enzymatic molecules like glutathione, uric acid, melatonin, and vitamins A, C and E, cannot inhibit the potent cytotoxic effects of those free radicals, and the oxidative balance of the organism shifts toward oxidative stress. Antioxidants are used up in detoxifying oxidant molecules, and the resultant oxidative stress is characterized by a decreased amount of antioxidants [8]. The increased oxidative stress causes oxidative damage to certain macromolecules of the organism, namely the proteins, lipids and the DNA. Recent studies demonstrated a strong correlation between oxidative stress and DNA damage [9,10].

Various methods are being used for monitoring DNA damage in peripheral mononuclear leukocytes, which is an indicator of the overall DNA damage in the body. Micronucleus (MN) test, chromosomal aberration analysis, the sister-chromatid exchange (SCE) test, gene mutation tests, and the comet assay test can be cited among such methods [11]. The comet assay (single-cell electrophoresis) test is a simple, rapid and sensitive test, and has a widespread use for determining the extensiveness of endogenous DNA damage [11,12]. Total oxidative status (TOS) and total antioxidant status (TAS) are parameters used widely for assessing the oxidative balance. Since measuring various oxidant and antioxidant molecules one by one is not practical, those parameters are used instead, and determine the total oxidant and anti-oxidant status [13,14].

There are numerous studies in medical literature focused on elucidating the pathophysiological basis of PFAPA syndrome, while no research has yet investigated the oxidative balance and DNA damage in such cases. Our study aims to explore DNA damage and the oxidative balance in patients clinically diagnosed with PFAPA.

2. Materials and methods

2.1. Subjects

Our study was conducted during March 2013 and January 2014, after obtaining the approval of the Local Clinical Research Ethics Committee. This cross-sectional study included 31 patients who were diagnosed with PFAPA (Group 1: PFAPA) in our university (Ear Nose and Throat and Pediatrics Clinics), 22 patients diagnosed with normal tonsillitis or pharyngitis (Group 2: NTP), and 20 healthy volunteers with similar demographic profiles (Group 3: CNT). The study design complied with the Helsinki Declaration principles. The study design was explained to the parents of all patients and volunteers, and written consents were obtained from all. Participants with chronic disorders, acute bacterial infection or other autoimmune diseases were excluded.

Patients who complied with the diagnostic criteria proposed by Thomas et al. [2] and accepted by the international medical community were included in the patient group of the study (Table 1). Patients were followed up for at least three periods of febrile episodes. Their demographics, clinical information and laboratory data were recorded. Patients who had infections or diagnosed with any other periodic fever syndrome were excluded from the study. Blood samples were drawn during the febrile episodes.

Twenty-two patients who seek for medical advice from Ear, Nose and Throat Department clinics in our university were diagnosed with tonsillitis or pharyngitis, included to the Group 2. These patients had no aphthous stomatitis, periodic episodes of fever, and cervical adenitis.

They did not have any other systematic diseases.

Table 1
Diagnostic criteria of the PFAPA syndrome.

Diagnostic criteria for Marshall's/PFAPA syndrome [2]
1. Regularly recurring fevers with an early age of onset (<5 years of age)
2. Symptoms in the absence of upper respiratory tract infection with at least one of the following clinical signs:
(a) Aphthous stomatitis
(b) Cervical lymphadenitis
(c) Pharyngitis
3. Exclusion of cyclic neutropenia
4. Completely asymptomatic interval between episodes
5. Normal growth and development

The control group (CNT) consisted of 20 healthy volunteers who did not have a history of PFAPA or a history of any other disease and/or drugs (during the past month). The volunteers were children who were being followed up by our University's Healthy Child Clinic, and were randomized from routine control appointments. Their demographics, clinical information and laboratory data were recorded. Blood samples were drawn from peripheral venous veins.

2.2. Blood collection and storage conditions

Five ml venous blood samples were drawn from the forearms of all participants. Blood samples were immediately drawn into heparinized tubes and were stored in dark, at a temperature of 2–4 °C, and were processed within 2 h to avoid any DNA damage due to hemolysis. Isolation of mononuclear leukocytes was conducted by centrifugation with Histopaque 1077 (Sigma). One ml of the heparinized blood specimen was carefully laid over Histopaque, and centrifuged at 500 × *g* for 35 min, at 25 °C. The interface band with the mononuclear leukocytes was centrifuged again with phosphate-buffered saline (PBS), at 400 × *g* for 15 min. Membrane integrity was measured with Trypan-Blue exclusion assay, and samples with over 90% cell viability were used for assessing DNA damage. The remaining heparinized blood specimens were centrifuged at 1500 × *g* for 10 min, and the plasma thus obtained was stored in −80 °C for the TOS and TAS analyses.

2.3. Determination of DNA damage using the alkaline comet assay

The comet assay, or the single-cell gel electrophoresis (SCGE) assay, was conducted with some modifications of the Singh et al.'s [11,15] method. Approximately 10 μl of fresh mononuclear leukocyte cell suspension (approx. 20,000 cells) was mixed with 80 μl of 0.7% low melting-point agarose in PBS at 37 °C. Next, 80 μl of each mixture was layered onto a slide precoated with a thin layer of 1% normal melting-point agarose (NMA) and immediately covered with a cover slip. Slides were held for 5 min at 4 °C to allow the agarose to solidify. After removal of cover slips, the slides were immersed in freshly prepared cold (4 °C) lysing solution (2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris–HCl, 1% Triton X-100, and 10% DMSO; pH 10–10.5) for at least 1 h. Triton X-100 and DMSO were added to the solution just before use. Slides were next immersed in freshly prepared alkaline electrophoresis buffer (0.3 M NaOH and 1 mM Na2EDTA; pH > 13) at 4 °C to allow DNA to unwind (40 min) and then electrophoresed (25 V/300 mA, 25 min). All manipulations were performed under minimal illumination. After electrophoresis, the slides were neutralized (0.4 M Tris–HCl; pH 7.5) for 5 min. Dried microscope slides were stained with ethidium bromide (2 mg/ml in distilled water; 70 μl/slide), covered with cover slips, and viewed by fluorescence microscopy (Olympus BX51, Japan) at 200x magnification. The microscope was capable of detecting epifluorescence and was equipped with a rhodamine filter (excitation wavelength 546 nm; barrier 580 nm).