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RESEARCH ARTICLE

Measurement of Serum Ceruloplasmin, Lipid Hydroperoxide Level and Prolidase Activity in Children with Primary Headache

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Abstract:

***Introduction*:** Primary headache is a significant health problem in children as it remarkably negatively affects the child and his/her family. Migraine and tension-type headaches constitute the majority of primary headaches in childhood. Studies regarding adult migraine patients suggest that oxidative stress has a significant role in the pathogenesis. This study aimed to investigate the relationship between primary headache in chidhood and the levels of oxidative stress markers.

***Materials and Methods*:** Pediatric patients diagnosed with primary headache and healthy controls in the pediatric age range were recruited. Data regarding age, gender, height, weight, and body mass index (BMI) were recorded. The levels of ceruloplasmin, lipid hydroperoxide, and prolidase activity were measured in plasma using the ELISA method. Statistical analyses were conducted using the SPSS 11.5 statistical program. A p-value of less than 0.05 was considered significant.

***Results*:** The study included 76 patients with primary headache and 61 healthy controls. The mean ages of the patients and healthy controls were 14.4±3.2 and 13.6±2.9 years. The patient and control groups were similar in terms of gender distribution (p=0.948), age (p=0.079), and BMI (p=0.196). Migraine accounted for 35.5% (n=27), while tension-type headache accounted for 64.5% (n=49) of the patients. Serum ceruloplasmin (p=0.033), lipid hydroperoxide (p<0.001), and prolidase (p=0.010) levels were higher in patients compared to the control group. Lipid hydroperoxide (p=0.021) and prolidase (p=0.013) levels were higher in migraine patients than in tension-type headache patients, while ceruloplasmin levels were similar between patients with different headache types (p=0.581).

***Conclusion*:** In this study, oxidative stress markers were shown to be increased in pediatric patients with primary headache. These findings support the hypothesis that patients with primary headaches are exposed to oxidative stress. Future studies may elucidate the role of oxidative stress in the etiopathogenesis of childhood migraine and other headache types.

Keywords: Headache, Oxidative stress, Migraine, Tension-type, Ceruloplasmin, Lipid hydroperoxide, Prolidase.

1. INTRODUCTION

Headache is a common health problem in both young children and adolescents [1-3]. The prevalence of headache increases throughout childhood and peaks at 11-13 years old [4]. Migraine and tension-type headaches (TTH) frequently constitute the primary headaches (PH) specific to the nervous system [5].

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Migraine has been reported to occur in 6.1-13.6% of children and TTH in 9.8-24.7%. Although the prevalence is estimated to be high, clinical data on PH in childhood are very limited. Most of the available data are compiled from studies on adult headaches.

Childhood headaches should be considered a significant health problem as they significantly negatively affect the child and his/her family [6,7]. Compared to children without headaches, these patients are more likely to miss school, have poorer peer relations, and have a lower quality of life [5].

Factors defined as risk factors or triggers associated with migraine and TTH include obesity, sleep disorders, behavioral and psychiatric factors, and dietary causes [8-10]. In addition to these triggers, oxidative stress has recently been thought to be associated with PH.

The oxidant-antioxidant balance is of great importance for maintaining homeostasis [11]. In physiological conditions, reactive oxygen radicals are continuously produced in all aerobic organisms and used for physiological purposes. At the same time, the antioxidant system triggered by oxidants keeps this balance under control. If the oxidant-antioxidant balance is disturbed in the direction of the oxidant system, oxidative stress emerges and leads to several disease processes.

Ceruloplasmin, involved in copper metabolism, is frequently associated with Wilson's disease [12]. However, it has been reported to be involved in plasma redox reactions and inhibit lipid peroxidation. Therefore, elevated plasma ceruloplasmin levels are accepted as an indicator of increased oxidative stress.

Since the literature has not adequately evaluated the relationship between oxidative stress and PH in the pediatric age group, our study aimed to compare prolidase activity, lipid peroxidation, and ceruloplasmin levels in children with PH and healthy controls.

2. MATERIALS AND METHODS

This descriptive study was conducted with patients younger than 18 who were diagnosed with PH in the Department of Pediatric Neurology at Harran University Faculty of Medicine and a healthy control group. Ethics committee approval for the study was obtained from the Harran University Faculty of Medicine Scientific Research Ethics Committee (21.12.2016/16217). Children whose families did not consent to the study, patients diagnosed with secondary headache, patients with chronic morbidities in addition to PH, and individuals with an acute disease when the blood sample was taken for the study purposes were omitted. Healthy controls were selected among children brought to Harran University Faculty of Medicine, Healthy Children Outpatient Clinic.

Sociodemographic information such as age, gender, height, weight, body mass index (BMI), and anthropometric characteristics of all participants were recorded in an electronic database. The PH type (migraine or TTH) and the frequency of attacks were also included in the database.

Venous blood samples were collected from all study participants to analyze serum ceruloplasmin, lipid peroxide, and prolidase activity. After the venous blood samples were centrifuged at 3500 rpm for 10 minutes, the shaped elements were discarded with the tube, and the remnants were stored at -800 C for analysis.

2.1. Measurement of Ceruloplasmin Level

In our study, ceruloplasmin levels were evaluated using the ELISA method. The analysis was performed according to Elabscience (CP) Ceruloplasmin ELISA Kit protocol: The lyophilized standard was centrifuged at 10.000xg for 1 min, 1ml Reference Standard & Sample Diluent was added and kept for about 15 min to be completely homogeneous. The standards were diluted 7-fold by serial dilution method (40-20-15-2.5-1.25-1.25-0.63-0 µg/mL). Prepared standards and serum samples were added 100µl to each well and incubated at 37°C for 90 min. After incubation, the liquid was removed and Biotinylated Detection Ab. (1:100) was added and incubated at 37°C for 60 min. All wells were washed 3 times with washing solution carefully. 100 µl HRP Conjugate (1:100) was added and incubated at 37°C for 30 min. All wells were carefully washed 5 times with the washing solution. 90 µl Substrate Reagent was added and

incubated at 37°C for 15 min (in the dark). 50 µl Stop Solution was added to stop the enzyme activity, and absorbance at 450nm was measured.

2.2. Measurement of Lipid Peroxide Level

The lipid peroxide levels were evaluated using the ELISA method. The analysis was performed according to the Cusabıo (LPO) Lipid Peroxide ELISA Kit protocol: The lyophilized standard was centrifuged at 10.000xg for 1 min, 1ml Standard Diluent was added and kept for about 15 min to be completely homogeneous. Standards were diluted 7-fold by serial dilution method (1000-500-250-150-125-62.5-31.25-15.6-0 ng/mL). 100µl of prepared standards and serum samples were added to each well and incubated at 37°C for 120 min. After incubation, the liquid was removed, and Biotin-antibody 1X was added and incubated at 37 °C for 60 min. All wells were washed 3 times with washing solution carefully. 100 µl HRP Avidin 1X was added and incubated at 37 °C for 60 min. All wells were washed 3 times with washing solution. 90 µl TMB Substrate was added and incubated at 37 °C for 15-30 min (in the dark). 50 µl Stop Solution was added to stop the enzyme activity, and absorbance at 450nm was measured.

2.3. Measurement of Prolidase Activity

The prolidase activity was analyzed using the ELISA method. The analysis was performed according to the Elabscience (PEPD) Peptidase D ELISA Kit protocol: The lyophilized standard was centrifuged at 10,000xg for 1 min. Then, 1 ml of Reference Standard & Sample Diluent was added and kept for about 15 min to make it completely homogeneous. The standards were diluted 7-fold by serial dilution method (200-100-50-25-12.5-6.25-3.13-0 ng/mL).

100µl of the prepared standards and serum samples were added to each well and incubated at 37 °C for 90 min. After incubation, the liquid was removed and Biotinylated Detection Ab. (1:100) was added and incubated at 37°C for 60 min. All wells were washed 3 times with washing solution carefully. 100 µl HRP Conjugate (1:100) was added and incubated at 37 °C for 30 min. All wells were carefully washed 5 times with the washing solution. 90 µl Substrate Reagent was added and incubated at 37 °C for 15 min (in the dark). 50 µl Stop Solution was added to stop the enzyme activity, and absorbance at 450nm was measured.

2.4. Statistical Analysis

Statistical analyses were performed using the SPSS version 20.0 (IBM® Inc, Chicago, USA) package program. Descriptive data were given as numbers, percentages, means, and standard deviations. The conformity of the variables to normal distribution was examined using visual (histogram and probability graphs) and analytical methods (Kolmogorov-Smirnov, Shapiro-Wilk tests). The numerical variables were compared between two groups using the Student t-test, and the One-Way ANOVA test was used to compare three groups. The homogeneity of variances was evaluated using Levene's test. Post-hoc analyses were performed with the Bonferonni test when significant differences were found. Pearson and Spearman correlation tests were used in correlation analysis. Numerical variables that did not show normal distribution were compared between two groups using the Mann-Whitney U test, and three or more groups were compared using the Kruskal Wallis Test. Chi-square analysis was used to compare ordinal data. A p-value below 0.05 was considered statistically significant.

3. RESULTS

Power analysis indicated that the sample size should be at least 120 patients. A total of 76 patients, 27 (35.5%) males, and 49 (64.5%) females were included in the study (Table **1**). The male/female ratio was 1.8:1. The control group comprised 61 healthy individuals, 22 (36.1%) males and 39 (63.9%) females. The male/female ratio was 1.7:1. The mean age was 14.4±3.2 years in the patient group and 13.6±2.9 years in the control group. The mean BMI was 19.1±2.4 kg/m2 (median 19.3, range 14.8-25.4) in the patient group and 18.5±2.9 kg/m2 (median 17.8, range 14.3-25.8) in the control group. There was no statistically significant difference between the patient and control groups in terms of gender, age and BMI

(p=0.948, p=0.079, p=0.196). While 35.5% (n=27) of the patients with headaches had migraines, 64.5% (n=49) had TTH.

Table 1. Demographic data.

-	Patient Group	Control Group	p Value
Age (year)*	14.4±3.2	13.6±2.9	0.079
Height (cm)*	147±17	142±15	0.081
Body weight (kg)*	43±13	39±14	0.104
Body-mass index*	19.1±2.4	18.5±2.9	0.196
Gender **	-	-	-
Male	35.5%	36.1%	0.948
Female	64.5%	63.9%	-

Note: *Independent t-test
**Chi-square test

The frequency of headache attacks was 10 or more per month in 30.3% (n=23) and less than 10 attacks per month in 69.7% (n=53) of the patients.

The serum ceruloplasmin level was 23.2±9.1 μg/mL in the patient group and 16.7±5.4 μg/mL in the control group. The ceruloplasmin level of the PH patients was statistically significantly higher than that of the control group (p=0.033) (Table **2**).

Table 2. Comparison of serum ceruloplasmin, lipid hydroperoxide and prolidase levels between two groups.

-	Patient Group	Control Group	p Value
Ceruloplazmin* (μg/ mL)	23.2±9.1	16.7±5.4	0.033
Prolidase* (ng/ mL)	94.2±12.3	58.8±11.5	0.010
Lipid hydroperoxide* (ng/ mL)	798.9±16.7	419.6±14.3	<0.001

Note: *Student t-test was used

The mean serum prolidase activity was 94.2±12.3 ng/mL in the patients and 58.8±11.5 ng/mL in the control group. The serum prolidase activity of the PH patients was statistically significantly higher than that of the control group participants (p=0.010).

The mean serum lipid hydroperoxide level was 798.9±16.7 ng/mL in the patients and 419.6±14.3 ng/mL in the control group. The serum lipid hydroperoxide level of the patients was statistically significantly higher than the control group (p<0.001).

There was no difference in serum ceruloplasmin levels between migraine and TTH (p=0.581). However, serum prolidase and lipid hydroperoxide levels were significantly higher in migraine headache group than in TTH group (p=0.021, p=0.013).

In migraine patients with a high number of attacks (≥10 attacks per month), serum ceruloplasmin level was 23.7±9.4 μg/mL, lipid hydroperoxide level was 939±25 ng/mL, and prolidase activity was 99.2±12.3 ng/mL; in migraine patients with a low number of attacks (<10 attacks per month), serum ceruloplasmin

level was 25.2±8.4 μg/mL, lipid hydroperoxide level was 723±23 ng/mL and prolidase activity was 84.7±9.7 ng/mL.

In patients with TTH with a high number of attacks (≥10 attacks per month), serum ceruloplasmin level was 28.2±10.4 μg/mL, and lipid hydroperoxide level was 756±23 ng/mL. Prolidase activity was 89.8±8.3 ng/mL in patients with TTH with a low number of attacks (< 10 attacks per month), serum ceruloplasmin level was 26.9±8.9 μg/mL, lipid hydroperoxide level was 723±18 ng/mL and prolidase activity was 86.7±9.2 ng/mL.

When the patients were grouped according to the frequency of attacks, the levels of lipid hydroperoxide and prolidase were higher in migraine patients with a high frequency of attacks than in migraine patients with a low frequency of attacks; however, ceruloplasmin levels did not differ ($p<0.001$, $p=0.036$, $p=0.354$). In patients with TTH, ceruloplasmin, lipid hydroperoxide, and prolidase levels were similar between patients with more and less frequent attacks ($p=0.668$, $p=0.656$, $p=0.913$).

4. DISCUSSION

Headache affects a significant number of children as well as adults [13]. By the age of 20, headache has been reported in 58% of the population. It is also the most common reason for referral to neurology clinics. Despite this fact, it may be neglected by families, teachers, and primary caregivers. Neglecting causes problems in social communication and school life and decreases quality of life [14]. Therefore, diagnosis and effective treatment of the disease is important. Although it is a common clinical entity, data on the pathogenetic mechanisms underlying PH are limited.

Although many studies have been conducted on the etiology of migraine, its pathogenesis still needs to be fully elucidated [15]. It has been suggested that oxidative stress, which occurs when the balance between the production of reactive oxygen products and antioxidant defense mechanisms is disrupted, is associated with various headache disorders, such as migraine [16].

Previous studies examined the potential role of oxidative stress in PH [17,18]. In most of these studies, oxidative stress levels were evaluated using various oxidant-antioxidant markers. Most studies were conducted in the adult age group, while studies evaluating oxidative stress levels in childhood headaches are limited [19].

Therefore, our study examined serum ceruloplasmin and lipid hydroperoxide levels in migraine and TTH. Their distribution in different PH types was also evaluated and compared with the control group. Prolidase enzyme activity, which has a vital role in collagen metabolism, was also evaluated in the patient and control groups [20]. The first striking finding of our study was that serum ceruloplasmin and lipid hydroperoxide levels and prolidase activity were higher in patients with PH compared to the control group. In addition, there was no difference in serum ceruloplasmin levels between patients with migraine and TTH. In contrast, migraine patients had higher serum prolidase activity and lipid hydroperoxide levels.

Lipid hydroperoxide is one of the most common lipid peroxidation products and an essential oxidative stress marker [18,21]. The most important effect of lipid peroxidation is the disruption and destruction of cell membranes. The brain is more sensitive to products released due to lipid peroxidation than other organ systems [22]. Studies investigating the role of oxidative stress in the etiology of headaches have shown high levels of malondialdehyde (MDA), the end product of lipid peroxidation [17,23,24].

Data on the level of lipid hydroperoxide in migraine patients are minimal [18]. In our study, lipid hydroperoxides were thought to be associated with PH, and lipid hydroperoxide levels were found to be higher in PH than in the control group. In light of our findings, it can be stated that lipid peroxidation increases in migraine patients, and oxidative stress, which is the expected result of lipid peroxidation, is at a higher level in these patients.

Ceruloplasmin, which is primarily involved in copper metabolism, controls lipid peroxidation [25]. Therefore, increased ceruloplasmin levels are an indicator of oxidative stress load [25,26].

The relationship between PH and serum ceruloplasmin was not examined before. In our study, the serum ceruloplasmin level was higher in PH than in the control group, which was thought to be an indirect indicator of oxidative stress. However, our findings should be confirmed by prospective controlled studies.

In a study by Erol *et al.,* antioxidant enzyme levels were evaluated in pediatric migraine patients [27]. This study found that catalase and glutathione peroxidase activities were lower in migraine patients than controls. As a result, it was concluded that oxidative stress played a significant role in the pathogenesis of pediatric migraine.

In a study by Gupta *et al.,* oxidative stress levels were evaluated in migraine and TTH patients and compared with the control group [17]. This study used FRAP (ferric reducing activity of plasma) and MDA as oxidative stress markers. The authors observed that MDA and FRAP levels were higher in migraine patients compared to the other two groups, while no difference was observed between TTH patients and the control group. In our study, oxidative stress levels were found to be higher in patients with migraine than in patients with TTH.

In a study by Vurucu *et al.,* 38 children with chronic daily headaches were evaluated [28]. The study showed that SOD, catalase, MDA, and GPx levels were higher in children with chronic daily headaches compared to controls.

In a recent study by Bernecker *et al.,* MDA and 4-hydroxy-2-nonenal (HNE) levels, which are lipid peroxidation products, were evaluated in 96 migraine patients [29]. While MDA levels were not found to be different, HNE levels were found to be higher in migraine patients than in controls.

It was also reported by Shimomura *et al.,* Ciancerelli *et al.,* and Tozzi-Ciancerelli *et al.* that oxidative stress levels were higher in migraine patients compared to controls [23,30,31].

On the other hand, some studies reported contradictory results regarding the potential association between migraine and oxidative stress [16,19,32]. A study by Geyik *et al.* analyzed the total oxidant level, total antioxidant level, and oxidative index in 50 migraine patients [16]. This study concluded that the oxidative stress level was not different from that of controls. Similarly, Eren *et al.* examined the same parameters in 141 migraine patients, but no difference was found between migraine patients and the control group [32].

Our study also analyzed the relationship between the frequency of attacks and oxidative stress. Lipid hydroperoxide and prolidase levels were higher in the group with a higher frequency of attacks. These results suggest that these markers can be used not only to diagnose disease but also as an indicator of disease severity.

Prolidase, a member of the metalloproteinase family, plays an important role in collagen synthesis [33]. It is found in leukocytes, erythrocytes, and keratinocytes, as well as in plasma. In their study conducted in 2017, Aslan *et al.* reported that serum prolidase activity was associated with oxidative stress [33]. However, to our knowledge, the role of prolidase in PH was not examined before. Based on its association with oxidative stress, serum prolidase activity was evaluated in PH for the first time in our study, and serum prolidase activity was higher in PH patients than in the control group.

Our study has some limitations. First, the number of patients included in our study is relatively small. On the other hand, most studies analyzed the levels of oxidative markers such as MDA, FRAP, total antioxidant level, total oxidant level, oxidative stress index, or HNE to elucidate the relationship between oxidative stress and migraine [16,17,29]. In our study, prolidase, ceruloplasmin, and lipid hydroperoxide, which have limited data, especially for pediatric patients, were preferred as the studied oxidative stress markers.

In our study, it was shown that oxidative load was higher in migraine patients. However, with these results, it cannot be fully understood whether increased oxidative stress causes migraine or migraine causes oxidative stress.

CONCLUSION

Oxidative stress is a function the human body requires to maintain its physiological life. It is kept under control by the antioxidant system. Disruption of this balance in favor of oxidants constitutes oxidative stress, blamed for numerous disease processes.

In our study, we found that prolidase, lipid hydroperoxide, and ceruloplasmin levels increased compared to controls. In addition, lipid hydroperoxide and prolidase levels were higher in migraine than in GTB. In light of these findings, it can be stated that oxidative stress has an important role in PH, especially in migraine type.

AUTHORS' CONTRIBUTIONS

The author confirms sole responsibility for the following: study conception and design, data collection, analysis and interpretation of results, and manuscript preparation.

CONSENT FOR PUBLICATION

Not applicable.

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None.

CONFLICT OF INTEREST

The author confirms that this article's content has no conflict of interest.

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