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Original Article

Oxidative stress in chronic headaches: old and new markers

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ABSTRACT

Objective: To study the effects of free radicals' overproduction in chronic primary headaches, we have evaluated two oxidative stress biomarkers, 3-nitrotyrosine (3-NT) and 8-hydroxy-deoxyguanosine expressed as ratio *vs* 2-deoxyguanosine (8-OHdG/2-dG). **Methods:** Analyses were done in plasma of three groups of subjects: chronic migraine (CM), chronic tension-type headache (CTTH) and healthy controls (CNT). 3-NT was analyzed by ultraviolet high-performance liquid chromatography (HPLC-UV), and 8-OHdG and 2-dG by two similar HPLC methods with electrochemical and fluorimetric detection, respectively. **Results:** In comparison to CNT group, 3-NT levels in plasma were significantly higher in CM patients whereas in CTTH were in the same range of healthy subjects. Instead, the mean 8-OHdG/2-dG ratios were higher in both chronic headache groups than CNT but without statistical significance (P values 0.46 and 0.1 for CTTH and CM, respectively). **Conclusion:** The higher mean plasmatic levels of 3-NT in CM only, suggest that just in this type of migraine an anomalous production of reactive nitrogen species occurs.

KEY WORDS: 2-Deoxyguanosine, 3-nitrotyrosine, 8-hydroxy-deoxyguanosine, chronic migraine, chronic tension-type headache, headache

INTRODUCTION

Chronic forms of primary headaches include chronic migraine (CM) and chronic tension-type headache (CTTH). Although the International Headache Society (IHS) has defined the criteria for the diagnosis of CM, the optimal definition of this primary headache is still a debating question, particularly when the patients use large amounts of acute drugs that lead to medication overuse [1-4].

The major requirement for the diagnosis of CM include the presence of headache for at least 15 days per month and a history of previous typical migraine attacks, with a portion of current attacks being classified as migraine without aura (MWoA) [3].

The prevalence of CM in the European population is quite high, being estimated in the range of 1.4 to 2.2%. In addition, 3% of episodic migraine sufferers and 14% in clinical cohorts may develop CM every year [5-6]. The social burden imposed by CM is relevant in terms of reduced health-related quality of life, increasing medical costs and limitations of daily living activities [7-8].

Similarly, the IHS diagnostic criteria for CTTH include presence of headache for at least 15 days per month being classified as typical tension-type headache attacks [2]. The prevalence of CTTH is reported to be around 14% of the general population.

Although the pathogenesis of migraine and its chronic process are still under investigation, it has been suggested that an increased level of oxidative stress products and consequent redox imbalance may play a significant role. However, the origin of these products is uncertain; it is possible that, during migraine attacks, an increased production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) may occur [9]. Particularly interesting is the role of nitric oxide (NO), an important mediator of neurogenic cranial vessel inflammatory response generated in endothelial cells. Enhanced endothelial NO release may cause changes in cerebral blood flow that ultimately might result in migraine.

Possibly, the frequency of attacks over times may cause migraine chronicity through an accumulation of RNS and ROS and a progressive deterioration of mitochondrial and neuronal functions. Given the difficulty in direct analysis of ROS and RNS due to their high reactivity and short half-life, previous studies on oxidative stress in headache were mainly based on colorimetric analysis of total oxidant and antioxidant blood capacities and on activities of enzymes involved in redox reactions such superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT). With our study, we wanted take a step forward by considering more specific stress markers able to highlight molecular damages resulting from oxidative attacks.

For proteins, one of the most sensitive oxidative biomarkers is 3-nitrotyrosine (3-NT), a nitration product of tyrosine residues mediated by RNS such as peroxynitrite anion ($ONOO^-$) and nitrogen dioxide (NO_2); its determination may be particularly interesting in migraine owing to the implication of NO imbalance already described. Due to the ubiquity of the proteins and their presence in free

form as well as in tissue structures, the nitration of tyrosine induced by RNS was proposed as marker of early stages of the pathogenetic process.

The prolonged action of ROS, particularly of hydroxyl radical, may also progressively induce oxidative alterations on nuclear and mitochondrial DNA, as modified nucleobases such 8-hydroxy-deoxyguanine (8-OHdG), subsequent mutations and breaking of DNA double strand. During DNA repairing process and especially following the degradation of nucleic acids consequent to cell death, the modified nucleoside 8-OHdG is released in extracellular fluids. Thanks to its molecular stability and specificity, the 8-OHdG plasma concentration is one of the most reliable markers of systemic oxidative stress. An increase in its diagnostic sensitivity could be obtained with the contemporary assay of the not-hydroxylated nucleoside 2-deoxyguanosine (2-dG). Calculation of 8-OHdG/2-dG ratio could indeed reflect the real DNA oxidative damage because the 8-OHdG value becomes, in this way, independent to the speed of DNA turnover that could be altered by disease and may be subject-specific [10]. The evaluation of both 3-NT, 8-OHdG and 8-OHdG/2dG ratio in chronic migraine seemed us useful to clarify the free radical formation processes and their pathological effects.

SUBJECTS AND METHODS

Patients

A group of 21 CM (5 males and 16 females, mean age 35 ± 15) and 17 CTTH (8 males and 9 females, mean age 32 ± 9) patients presenting at Headache Center of Neurology Departments in three Italian hospitals (Vicenza, Milan and Asti) were enrolled. The inclusion criteria consisted of a history of chronic headaches lasting one year or more and a diagnosis of CM or CTTH [2-3].Because not responsive to prophylactic drug treatments, all the subjects were under symptomatic therapy only.

A cohort of 44 healthy subjects (22 males and 22 females, mean age 32 ± 11) was used as control group. Subjects with headaches, diabetes, hypertension or other relevant disorders were excluded. After obtaining informed consent, the levels of 3-NT, 8-OHdG and 2dG were measured in the plasma of all enrolled subjects with HPLC methods.

Biological samples

Blood was collected in vacuum sealed tubes containing EDTA and immediately centrifuged at 3500 rpm, for 10 min, at +4°C; plasma samples were frozen at -80°C until analysis. Both standards and plasma samples were processed according to the manufacturer's method for 3-NT and as previously described for 8-OHdG and 2-dG [10].

Chemicals and solutions

All chemicals were of analytical grade and purchased from Sigma-Aldrich (Milan, Italy). 3-NT was analyzed with a HPLC-UV kit from Eureka (Ancona, Italy). 8-OHdG and 2-dG assays were performed by two home-made HPLC methods as previously reported [10]. The cartridges for 8-OHdG and 2-dG solid phase extraction (MF C18 Isolute 50 mg) were purchased from Step-Bio (Bologna, Italy).

Stock solutions for 8-OHdG and 2-dG were prepared in water and kept at -80°C until analysis. One aliquot of each stock solution was adequately diluted, first in water and finally in plasma matrix pooled from donor samples to obtain working spiked solutions ranging from 0.01 to 0.5 and from 100 to 5000 ng/ml for 8-OHdG and 2-dG, respectively.

High-performance liquid chromatography analysis

The HPLC system consisted of a model 307 pump and a model 234 autosampler with 100 µl loop, both from Gilson (Middleton, WI, USA). For 8-OHdG and 2-dG assays, the separations were performed on a C18 column (Waters X-Bridge Shield 250 mm x 4.6 mm internal diameter) packed with 5 µ particles, with a precolumn (10 mm x 4.6 mm internal diameter) packed with the same material. The mobile phase was an aqueous solution of 25 mM formic acid with 7% acetonitrile, flushed at 1 ml/min. 8-OHdG was revealed with an electrochemical detector Coulochem II ESA fitted with a model 5011 high-sensitivity cell (first electrode +0.00 V, second +0.620 V); in a separate run, 2-dG was then analyzed with a fluorimetric detector model 920 Jasco (excitation and emission wavelengths 264 and 340 nm). The analytical column for 3-NT was an Agilent Poroshell 120 EC-C18 (50 mm x 4.6 mm internal diameter) packed with 2.7 µ particles and the UV detector a model 875-UV Jasco (wavelength 232 nm).

Statistical analysis

Stata 9.0 software was used; the non-parametric Wilcoxon rank sum test for unpaired data was employed to compare the medians of cases and controls. A significance level of 5% was always adopted ($P < 0.05$).

RESULTS

The employed HPLC methods allow us to analyze 8-OHdG, 2dG and 3-NT in relative short time and with high specificity, sensitivity and accuracy (Figures 1-2).

Both 8-OHdG and 2-dG did not show statistically significant differences between CNT, CTTH and CM patients, although lower values of 2-dG in CM than CNT were very close to the significance, fixed at 5% level (Tables 1-2). Even when considering the mean values of 8-OHdG/2-dG ratio, only little differences were found between CNT and migrainous patients, with mean ratios higher in both chronic headache groups than in controls (Figure 3). These differences were once again not statistically significant.

Instead, 3-NT main levels in plasma were much higher in CM than CTTH and controls (Tables 1-2, Figure 4) and the statistical analysis showed full significance ($P < 0.01$).

Table 1. Plasma levels of 3-NT, 2-dG, 8-OHdG and 8-OHdG/2-dG ratio in controls(CNT), chronic tension type headache (CTTH) and chronic migraine (CM) groups

	3-NT (ng/ml)		8-OHdG (pg/ml)		2-dG (ng/ml)		8-OHdG/2-dG ratio (pg / ng x10^3)	
	mean	SD	mean	SD	mean	SD	mean	SD
CNT (n = 44)	**17.3**	5.1	**129.1**	73.1	**775.5**	201.4	**154**	91.3
CTTH (n = 17)	**15.8**	8.5	**133.8**	112.6	**639.7**	77.1	**203.4**	188.4
CM (n = 21)	**47.4**	33.8	**132.8**	58.5	**673**	93.5	**208.3**	85.9

Table 2. P values for 3-NT, 2-dG, 8-OHdG and 8-OHdG/2-dG ratio between control (CNT), chronic tension-type headache (CTTH) and chronic migraine (CM) groups

	3-NT (ng/ml)	8-OHdG (pg/ml)	2-dG (ng/ml)	8-OHdG/2-dG ratio (pg / ng x10^3)
CNT *vs* CTTH	0.66	0.91	0.17	0.46
CTTH *vs* CM	**0.01****	0.98	0.48	0.93
CNT *vs* CM	**0.00****	0.86	**0.06***	**0.1***

**significantly different; *different, but not significant

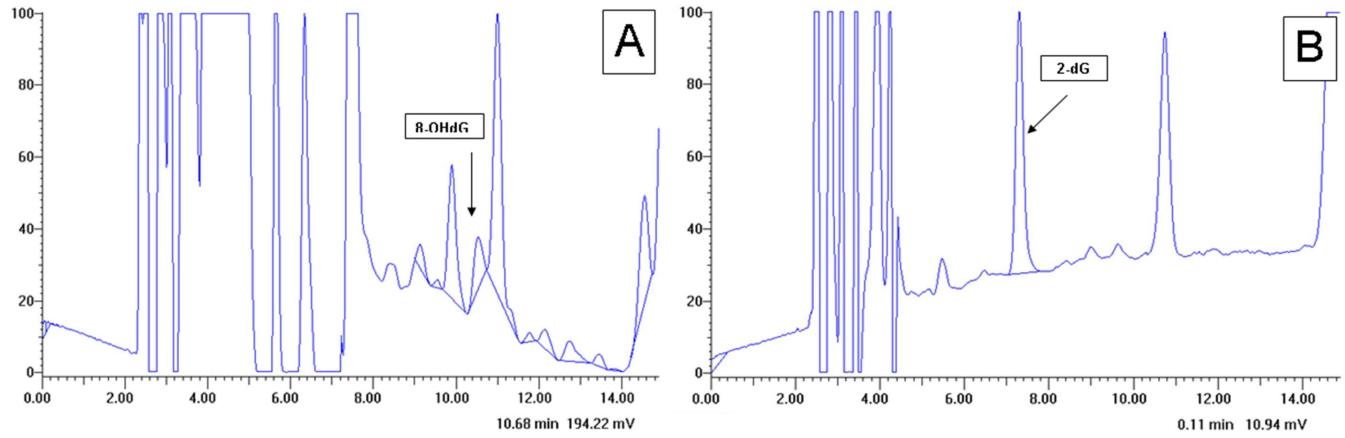

Figure 1. Typical chromatograms of a plasma extract in HPLC conditions for 8-OHdG (**A**) and 2-dG (**B**) respectively. X-axsis shows the chromatographic time in minutes and y-axsis the electrochemical and fluorimetric detector response in relative units.

DISCUSSION

Molecular mechanisms of migraine have not been fully clarified yet and little is known about the role of oxidative stress in the pathogenesis of primary headaches. It was previously reported that strong pro-oxidant species such as thiobarbituric acid reactive substances (TBARS) and nitrate were increased in plasma or urine of migraine patients, with (MWA) and without aura (MWoA), and were associated to platelet membrane alterations and functional abnormalities. These results seemed to indicate that an increased ROS exposition may develop a progressive impairment of platelet function in migraine [11].

Shimomura *et al* [12], showed that platelets activities of antioxidant enzymes such SOD decreased in MWA but not in MWoA and CTTH, suggesting the higher vulnerability to oxidative stress in MWA and its implication in the etiology. Tozzi-Ciancarelli *et al* [13] hypothesize that enhanced endothelium NO and superoxide anion release may cause migraine through changes in cerebral blood flow. To support this theory, they showed that urinary NO stable metabolites (NOx) and TBARS were higher in migraine patients than in a control group [14]; during migraine attack also, NOx and TBARS excretion were higher with respect to the headache-free period. By a preventive non-pharmacological treatment named *biofeedback*, it was possible to decrease migraine attacks influencing NO bioavailability in patients with CM and inducing changes in regional cerebral blood flow mediated by oxygen free radicals that react with NO. The efficacy of *biofeedback* treatment in migraine was even demonstrated in a successive study by determination of NOx, peroxide anion and SOD activity [15].

The association of an increased nitrosative and oxidative stress in migraine attacks was demonstrated also from Yilmaz *et al* [9] with analysis of platelets contents of nitrite and nitrate, as indicators of NO production, and

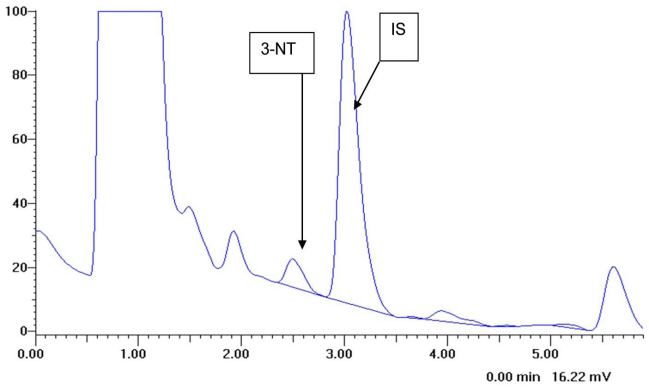

Figure 2. Typical chromatogram for 3-NT and IS in a plasma derivative sample. X-axsis shows the chromatographic time in minutes and y-axsis the UV-detector response in relative units (0.001 full scale). 3-NT quantification was performed by plasma-matrix calibration and internal standard (IS) correction.

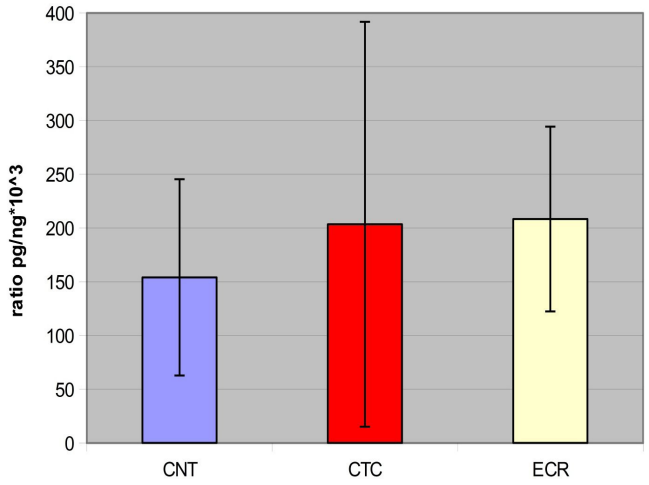

Figure 3. Graphic representation (mean ± SD) of plasma 8-OHdG/2-dG ratio in the three groups.

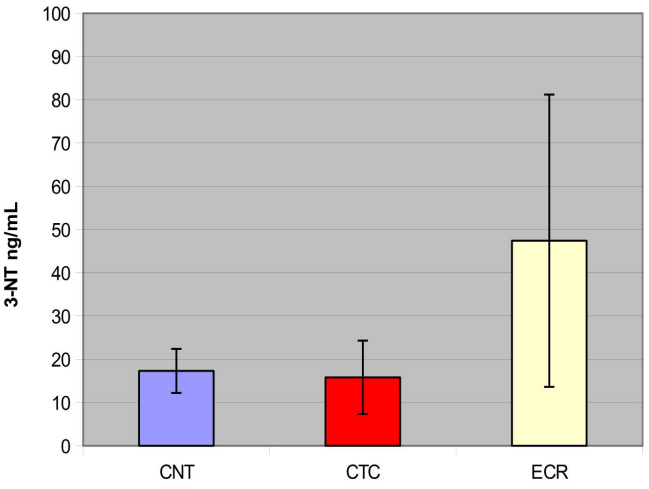

Figure 4. Graphic representation (mean ± SD) of plasma 3-NT levels in the three groups.

malondialdehyde (MDA) and total thiol levels as markers of oxidative status. Particularly the increase of NO activity in platelets during attacks supports the theory that it may play a modulation role on vasodilatation in migraine attacks.

Tuncel *et al* [16], for the first time, hypothesized the pathological role of an impaired mitochondrial oxidative metabolism in migraine. The MDA levels in CM were significantly higher than controls and SOD activity was significantly higher in MWA than in MWoA; instead, no correlation was found between oxidative stress markers and headache attack period. Cordero *et al* [17] remarked the role of mitochondrial dysfunction and oxidative stress in the headache symptoms associated with fibromyalgia. Decrements in coenzyme Q10, catalase and ATP levels in blood mononuclear cells have been found in fibromyalgia; an oral Q10 supplementations showed remarkable improvement in clinical symptoms and headache [18, 19]. More recently, it has been proposed that the observed increase of the elusive amines tyramine, octopamine and synephrine in MWoA and CM could result from a shift of tyrosine metabolism consequent of a mitochondrial damage caused by nitrosative and oxidative stress [20-21].

Studies on migraine and oxidative stress did not always give unequivocal results. Erol *et al* [22], studying the activities of erythrocyte SOD, GPX and CAT in children and adolescents with migraine, found that SOD did not differ between groups, while GPX and CAT were significantly lower in migraine patients than controls. Previously, Bockowski *et al* [23] published that serum and erythrocyte GPX were higher and erythrocyte SOD were decreased in migraine. More recently, Vurucu *et al* [24] investigated the relationship between oxidative stress and chronic daily headache in children finding that erythrocyte SOD, GPX and CAT were all higher in migraine than in control group.

An Indian comparative study between CM and CTTH based on analysis of plasma ferric reducing ability (FRAP) and MDA levels pointed out that tension headaches are not similar to migraines as regards the oxidative stress markers. CM showed highest values of MDA and FRAP while no differences were observed between CTTH and the control group [25].

In recent years, because the strong suggestion of an oxidative imbalance implication, the attention of researchers has focused on molecular pathogenetic alterations in migraine [26]. Ooi *et al* [27], studying the signaling role of NO in neurons of trigeminal ganglia, identified an action site on a triplet of cysteines: this molecular point could be easily a site of oxidative modification mediated by ROS.

So, a tight control of local redox status and NO enviroment could exercise a fine regulation on neural excitability and justify the pathogenetic role of oxidative stress. Even if the oxidative stress may be the cause or the effect of migraine, an antioxidant therapy was introduced in order to re-equilibrate an unbalanced redox status and, as consequence, to reduce severity and frequency of migraine [28]. Dominguez *et al* [29] have already studied organic

extracts of some plants used in folk medicine. Although neither the type of bioactive components or biochemical mechanisms involved have been assessed, this work showed that the extracts can effectively fight the formation of free radicals and TBARS. Chayasirisobhon *et al* [28] studied the benefits of *Pinus radiata* bark extract and vitamin C as treatment for migraine. The responders, who continuously took the bark extract and vitamin C combination for 12 months, experienced ongoing migraine relief with more than 50% reduction of frequency and severity of headaches. Similar conclusions are reported from other Authors who had studied traditional herbal remedies [30], antioxidant [31] and vitamin diet supplementations [32].

Therefore, the results previously reported, taken together, seem to suggest that migraine patients are under an imbalance of redox status due to a continuous nitrosative and oxidative stress generated during the migraine attacks. It was not known if these alterations are present either in CM or in CTTH and if these play a role in the chronic evolution.

In order to investigate the hypothesis of pathogenetic role of oxidative stress in migraine we studied two important markers of damage, 3-NT and 8HdG/2dG in plasma. By-passing the assessment of the effectiveness of oxidative attack and the efficacy of the antioxidant enzymatic and non-enzymatic barrier, it was so possible evaluate the true molecular damages caused by the increased production of ROS and RNS. With full statistical significance, the higher mean levels of 3-NT in plasma of CM patients, seemed to confirm that an abnormality of NO turnover was present in this migraine and that the elevated amount of nitrogen radical may play a role in the chronicity process. In contrast, the demonstration that 3-NT plasma levels in CTTH were in the same range of controls seemed to show that NO metabolism in CTTH was quite normal and that the pathogenesis mechanism differs from CM. These results, although in partial contrast with Van der Schueren *et al* [33], which showed no increments of endothelial NO synthase, are instead in good agreement with other reports [34].

The 8-OHdG/2dG ratio resulted slight altered both in CTTH and CM than in CNT, but without full statistical significance. While the mean concentrations of 8-OHdG were about the same for CNT, CTTH and CM, 2-dG was slightly lower in CTTH and CM than CNT. This could imply a less efficient nucleoside recovery pathway in migraine and a consequent decreased restoration of native nucleotides pool. From that decrease may result a slowdown of DNA repairing systems and the persistence of a higher oxidation damage on nucleotide chains. This higher DNA damage would in fact demonstrated by the increased 8-OHdG/2-dG mean ratios both in CTTH and CM than CNT, but the lack of statistical significance makes impossible to draw conclusions.

Anyway, the elevation of 8-OHdG/2-dG ratios both in CTTH and CM seemed to suggest that while the nitrosative damage characterized mainly CM, DNA oxidation affects both forms of primary headaches. Just because the headache attack is the leading cause of the overproduction of ROS and RNS, the scatter of our data and the probably consequential lack of full statistical significance could be depended on the elapsed time between headache attack and blood collection. We are so planning future studies more standardized respect this time.

Taken together, it was known that there is an imbalance between production and neutralization of free oxygen and nitrogen radicals in chronic migraine. The present data confirm that oxidative stress is a common condition in this pathology, since there is a slight increment of 8-OHdG/2dG ratio both in CM and CTTH compared to controls. However, the pathogenetic mechanism is probably different for CTTH and CM. Indeed, the higher mean plasmatic levels of 3-NT in CM only, strongly suggest that just in this type of migraine an anomalous production of RNS occurs. Further studies in this respect are certainly needed.

REFERENCES

1. Silberstein SD, Lipton RB, Sliwinski M. Classification of daily and near-daily headaches: field trial of revised IHS criteria. Neurology 1996; 47:871-87.
2. Headache Classification Subcommittee of the International Headache Society. The International Classification of Headache Disorders, 2nd Edition. Cephalalgia 2004; 24:9-160.
3. Headache Classification Committee, Olesen J, Bousser MG, Diener HC, Dodick D, First M, Goadsby PJ, Göbel H, Lainez MJ, Lance JW, Lipton RB, Nappi G, Sakai F, Schoenen J, Silberstein SD, Steiner TJ. New appendix criteria open for a broader concept of chronic migraine. Cephalalgia 2006; 26:742-6.
4. Bigal ME, Rapoport AM, Sheftell FD, Tepper SJ, Lipton RB. The International Classification of Headache Disorders revised criteria for chronic migraine-field testing in headache speciality clinic. Cephalalgia 2007; 27:230-4.
5. Manzoni GC, Torelli P. Proposal for a new classification of chronic headache. Neurol Sci 2010; 31:S9-13.
6. Manack AN, Buse DC, Lipton RB. Chronic migraine: epidemiology and disease burden. Curr Pain Headache Rep 2011; 15:70-8.
7. Scher AI, Stewart WF, Ricci JA, Lipton RB. Factors associated with the onset and remission of chronic daily headache in a population-based study. Pain 2003; 106:81-9.
8. Katsarava Z, Schneeweiss S, Kurth T, Kroener U, Fritsche G, Eikermann A, Diener HC, Limmroth V. Incidence and predictors for chronicity of headache in patients with episodic migraine. Neurology 2004; 62:788-90.
9. Yilmaz G, Surer H, Inan LE, Coskun O, Yucel D. Increased nitrosative and oxidative stress in platelets of migraine patients. Tohoku J Exp Med 2007; 211:23-30.
10. Bolner A, Pilleri M, De Riva V, Nordera GP. Plasma and urinary HPLC-ED determination of the ratio of 8-OHdG/2dG in Parkinson's disease. Clin Lab 2011; 57:859-66.
11. Tozzi-Ciancarelli MG, De Matteis G, Di Massimo C, Marini C, Ciancarelli I, Carolei A. Oxidative stress and platelet responsiveness in migraine. Cephalalgia 1997; 17:580-4.
12. Shimomura T, Kowa H, Nakano T, Kitano A, Marukawa H, Urakami K, Takahashi K. Platelet superoxide dismutase in migraine and tension-type headache. Cephalalgia 1994; 14:215-8.

13. Ciancarelli I, Tozzi-Ciancarelli MG, Di Massimo C, Marini C, Carolei A. Urinary nitric oxide metabolites and lipid peroxidation by-products in migraine. Cephalalgia 2003; 23:39-42.
14. Ciancarelli I, Tozzi-Ciancarelli MG, Di Massimo C, Olivieri L, Carolei A. Preventive non-pharmacological treatment and nitric oxide in migraine. J Headache Pain 2005; 6:341-2.
15. Ciancarelli I, Tozzi-Ciancarelli MG, Spacca G, Di Massimo C, Carolei A. Relationship between biofeedback and oxidative stress in patients with chronic migraine. Cephalalgia 2007; 27:1136-41.
16. Tuncel D, Tolun FI, Gokce M, Imrek S, Eckerbicer H. Oxidative stress in migraine with and without aura. Biol Trace Elem Res 2008; 126:92-7.
17. Cordero MD, Cano-Garcia FJ, Alcocer-Gomez E, De Miguel M, Sanchez-Alcazar JA. Oxidative stress correlates with headache symptoms in fibromyalgia: coenzyme Q10 effect on clinical improvement. PLoS One 2012; 7:1-6.
18. Edeas M, Weissig V. Targeting mitochondria: strategies, innovations and challenges. The future of medicine will come through mitochondria. Mitochondrion 2013; 13:389-90.
19. Finsterer J. Treatment of central nervous system manifestations in mitochondrial disorders. Eur J Neurol 2011; 18:28-38.
20. D'Andrea G, D'Arrigo A, Facchinetti F, Del Giudice E, Colavito D, Bernardini D, Leon A. Octopamine, unlike other trace amines, inhibits responses of astroglia-enriched cultures to lipopolysaccharide via a beta-adrenoreceptor-mediated mechanism. Neurosci Lett 2012; 517:36-40.
21. D'Andrea G, Leon A. Pathogenesis of migraine: from neurotransmitters to neuromodulators and beyond. Neurol Sci 2010; Suppl 1:S1-7.
22. Erol I, Alehan F, Aldemir D, Ogus E. Increased vulnerability to oxidative stress in pediatric migraine patients. Pediatr Neurol 2010; 43:21-4.
23. Bockowski L, Sobaniec W, Kulak W, Smigielska-Kuzia J. Serum and intraerythrocyte antioxidant enzymes and lipid peroxides in children with migraine. Pharmacol Rep 2008; 60:542-8.
24. Vurucu S, Karaoglu A, Paksu MS, Yesilyurt O, Oz O, Unay B, Akin R. Relationship between oxidative stress and chronic daily headache in children. Hum Exp Toxicol 2013; 32:113-9.
25. Gupta R, Pathak R, Bhatia MS, Banerjee BD. Comparison of oxidative stress among migaineurs, tension-type headache subjects, and a control group. Ann Indian Acad Neurol 2009; 12:167-72.
26. Alp R, Selek S, Alp SI, Taskin A, Kocyigit A. Oxidative and antioxidative balance in patients of migraine. Eur Rev Med Pharmacol Sci 2010; 14:877-82.
27. Ooi L, Gigout S, Pettinger L, Gamper N. Triple cysteine module within M-type K+ channels mediates reciprocal channel modulation by nitric oxide and reactive oxygen species. J Neurosci 2013; 33:6041-6.
28. Chayasirisobhon S. Efficacy of Pinus radiata bark extract and vitamin C combination product as a prophylactic therapy for recalcitrant migraine and long-term results. Acta Neurol Taiwan 2013; 22:13-21.
29. Dominguez N, Nieto A, Marin JC, Keck AS, Jeffery E, Cespedes CL. Antioxidant activities of extracts from *Barkleyanthus salicifolius* (Asteracee) and *Penstemon gentianoides* (Scrophulariacee). J Agric Food Chem 2005; 53:5889-95.
30. Bhandare A, Kshirsagar A, Vyawahare N, Sharma P, Mohite R. Evaluation of anti-migraine potential of *Areca catechu* to prevent nitroglycerin-induced delayed inflammation in rat meninges: possible involvement of NOS inhibition. J Ethnopharmacol 2011; 136:267-70.
31. Alstadhaug KB, Odeh F, Salvesen R, Bekkelund SI. Prophylaxis of migraine with melatonin: a randomized controlled trial. Neurology 2010; 75:1527-32.
32. Visser EJ. Is migraine a complex regional pain syndrome of the brain? Migraine prophylaxis with vitamin C? Pain Pract 2011; 11:199-200.
33. Van der Schueren BJ, Verbrugge FH, Verbesselt R, Van Hecken A, Bepre M, de Hoon JN. No arguments for increased endothelial nitric oxide synthase activity in migraine based on peripheral biomarkers. Cephalalgia 2010; 30:1354-65.
34. Gruber HJ, Bernecker C, Lechner A, Weiss S, Wallner-Blazek M, Meinitzer A, Hobarth G, Renner W, Fauler G, Horejsi R, Fazekas F, Truschnig-Wilders M. Increased nitric oxide stress is associated with migraine. Cephalalgia 2010; 30:486-92.

Conflict of Interest: None declared