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Increased Nitrosative and Oxidative Stress in Platelets of Migraine Patients

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YILMAZ, G., SÜRER, H., İNAN, L.E., COŞKUN, Ö. and YÜCEL, D. *Increased Nitrosative and Oxidative Stress in Platelets of Migraine Patients*. Tohoku J. Exp. Med., 2007, **211** (1), 23-30 —— The molecular mechanisms of migraine have not been fully clarified yet. Increased nitrosative and oxidative stress may be associated with migraine attacks. Platelets may play an important role in migraine patients and they can reflect the lability of tissues to nitrosative/oxidative stress. In the present study, we aimed to determine the levels of nitrosative and oxidative stress markers in platelets of migraine patients during headache-free and attack periods. A total of 56 subjects (22 migraine without aura, 14 migraine with aura, and 20 age- and sex-matched healthy controls) were included in the study and nitric oxide (NO) metabolites, malondialdehyde (MDA), and thiol (SH) groups were measured in platelets. During migraine attacks, platelet levels of nitrate, nitrite and MDA were significantly higher in migraineurs than these in control subjects ($p = 0.042$, $p = 0.005$ and $p = 0.042$, respectively). By contrast, during headache-free period, no statistically significant differences were found in the platelet levels of nitrate, nitrite and MDA between migraineurs and controls ($p > 0.05$), although the marginal increases were detected in migraineurs. These results suggest that increased biomarkers of nitrosative and oxidative stress in platelets may be important in migraine patients, especially during attacks; increase of NO metabolites in platelets during attacks supports the opinion that NO may play a modulatory role in biological processes particularly by vasodilatation in migraine attacks. Therefore, MDA and NO metabolites may serve as useful markers to show the increased vulnerability to nitrosative and oxidative stress in migraine patients. ——
headache; migraine; nitric oxide; oxidative stress; nitrosative stress

Migraine is a common neurovascular disorder characterized by attacks of severe headache and autonomic and neurological symptoms. The molecular mechanisms of migraine have not been fully clarified yet (Moncada et al. 1991). We hypothesize that increased oxidative and nitrosative stress may together take place in patients with migraine, especially during attacks.

Nitric oxide (NO), a labile molecule with a half life of only a few seconds, is synthesized mainly in the endothelium (Moncada et al. 1991). It is rapidly oxidized by tissue oxygen to the

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stable end products, nitrate (NO^{3-}) and nitrite (NO^{2-}). In the circulation, nitrite is almost converted to nitrate by hemoglobin (Stamler et al. 1992). The best index of overall NO production, therefore, is the total concentration of both nitrate and nitrite. NO has emerged as an important mediator of the initiation and possibly propagation of neurogenic cranial vessel inflammatory response that ultimately might result in headache. In cerebral vessels, NO might excite and sensitize perivascular afferents (Shukla et al. 2001). It was suggested that NO may play a modulatory role on biological processes such as vasodilatation in migraine attacks (Dawson et al. 1992).

It has been reported that patients suffering from migraine with aura have vulnerability to oxidative stress (Shimomura et al. 1994; Tozzi-Ciancarelli et al. 1997). It was also suggested that reactive free radicals may be stimulated during migraine attacks in amounts sufficient to overcome normally effective protective mechanisms (Tozzi-Ciancarelli et al. 1997). Malondialdehyde (MDA), one of the well-known secondary products of lipid peroxidation after exposure to reactive oxygen species and free radicals, may be used to evaluate oxidative damage (Esterbauer et al. 1991; Karatas et al. 2002). Plasma thiols are physiological free radical scavengers and they may serve an antioxidant function by several mechanisms. It was reported that the measurement of plasma total thiol (SH) is a good reflection of excess free radical generation both in physiological and pathological conditions in human (Hu 1994; Pasaoglu et al. 2004).

Blood platelets, the smallest blood cells, are involved in hemostasis, inflammation and thrombosis. They may also play an important role in migraine patients. Platelet dysfunction has been linked to the pathophysiology of migraine (Kitano et al. 1994; Taffi et al. 2005). Additionally, platelets can reflect the lability of tissues to nitrosative and oxidative stress.

Although above mentioned studies reported increase of separate markers of oxidative and nitrosative stress which were associated with migraine attacks, a systematic study reporting the status of whole of these markers in platelets has not been conducted previously in migraineurs with and without aura during headache-free and attack periods. In view of these thoughts, in the present study, we determined nitrite and nitrate levels as indicators of NO production, and MDA and total thiol levels as markers of oxidative status in the platelets of migraine patients with or without aura.

Patients and Methods

Patients

A total number of 56 subjects (22 migraine without aura [MWOA], 14 migraine with aura [MWA], and 20 healthy control) were included in the study. MWOA group was consisted of 2 male (M) and 20 female (F) subjects; the mean age was 29.0 ± 7.4 y and average illness duration was 4.7 y (range, 1 to 10 y). MWA group was consisted of 2 M and 12 F; the mean age was 32.5 ± 6.1 y and average illness duration was 5.7 y (range, 1 to 15 y). Control group was consisted of 20 healthy subjects (5 M and 15 F; the mean age was 30.1 ± 7 y) from laboratory staff. Diagnosis of all patients was made by a neurologist according to International Headache Society (IHS) criteria (Headache Classification Committee of the International Headache Society 1988). None of the patients had taken any drug for 10 days before blood sampling. Blood sampling was performed within 2-5 hrs from the onset of migraine attack. The characteristics of patients and controls are shown in Table l. All of the patients and controls were informed and the study was approved by Local Ethics Committee of Ankara Education and Research Hospital. All the investigators confirm to ethical standards as described in the Declaration of Helsinki. The study was performed in compliance with relevant laws and institutional guidelines.

Platelet isolation

A centrifugal isolation technique was used (Leeksma and Cohen 1956). Ten ml of whole blood was mixed with 1 ml of 1% (w/v) disodium ethylenediamine tetraacetic acid in 0.9% (w/v) saline, and was centrifuged at 65 g for 20 min. The supernatant (platelet rich plasma) was pipetted into another tube and centrifuged at 250 g for 20 min and the sedimented platelets were collected and washed twice with 0.9% (w/v) saline. The platelets were suspended in Hank's Balanced Salt Solution: (HBSS: sodium chloride 138 mM, potassium chloride 2.7 mM, disodium hydrogen phosphate 8.1 mM, potassium

TABLE 1. Clinical details of migraine patients and control subjects.

	MWOA	MWA	Controls
Gender (F/M)	20/2	12/2	15/5
Mean age (y)	29.0 ± 7.4	32.5 ± 6.1	30.1 ± 7.0
Family history (n)	5	3	
Migraine history (y)	4.7 ± 3.1	5.7 ± 4.4	
Annual frequency of attacks	31.6 ± 18.7	29.1 ± 16.1	
Mean duration of attacks (h)	33.1 ± 22.7	40.7 ± 22.0	

MWOA, Migraine without aura; MWA, Migraine with aura.

dihydrogen phosphate 1.5 mM, magnesium chloride 0.6 mM, calcium chloride 1.0 mM, glucose 10 mM, pH 7.4) and were counted using a Coulter Counter Gen S (Beckman – Coulter, Inc., Fullerton, CA, USA). Cells suspended in the HBSS were disrupted by a Heidolph DIAX 900 homogenizer (Heidolph Elektro, Kelheim, Germany) and centrifuged at 2,000 g for 20 min at 4°C, and supernatant was separated and stored at –20°C until working day. The stored platelet extracts were thawed and nitrite, nitrate, MDA and SH measurements were performed in these preperations in the same working day.

Nitrite/Nitrate, MDA and SH estimation

Nitrate and nitrite content in the platelets (1×10^8 cells/ml HBSS) obtained from patients with migraine and healthy age-matched controls were measured by the modified Griess method (Smarason et al. 1997). This method is based on the reduction of nitrate to nitrite by nitrate reductase and measurement of the occurred color in the end of the reaction of nitrite with Griess reagent; nitrite alone was then measured without enzymatic reaction and nitrate was calculated by substraction; the resultant color changes were read at 540 nm; calibration curves were made with sodium nitrite and potassium nitrate standards prepared in deionised water. The method developed by Hunter et al. (1985) which detects lipid peroxidation products generated by peroxidative processes with thiobarbituric acid (TBA) is used for MDA determination. Briefly, 250 μl of homogenized isolate and 250 μl of 35% trichloroacetic acid were mixed in glass tubes and 250 μl Tris – HCl buffer (pH 7.4) was added; after addition of 500 μl of 0.75% TBA in 2 M Na_2SO_4 and mixing, all the tubes were heated in a water bath at 95 – 100°C for 45 min. After the samples cooled, 500 μl of 70% trichloroacetic acid was added, the tubes were centrifuged at 950 g for 10 min and absorbances of supernatants were read at 530 nm against a water blank. The calibration curve was prepared with 0-25 μmol/l malondialdehyde standards, which were generated by hydrolysis of 1,1′,3,3′-tetraethoxypropane. SH groups were measured by a colorimetric method that thiol groups give a chromogen compound with dithiobisnitrobenzoic acid (DTNB) in alkaline pH (Sedlak and Lindsay 1968). In brief, aliquots of 200 μl of the platelet homogenates were mixed with 600 μl of 0.2 M Tris buffer, pH 8.2, and 40 μl of 0.01 M DTNB in methanol. After the addition of 3.16 ml of absolute methanol, the test tubes were allowed to stand for 15 min and then centrifuged at 3,000 g at room temperature for 15 min. The absorbances of supernatants and a reagent blank (without sample) and a sample blank (without DTNB) were measured at 412 nm. SH concentrations were calculated by the use of molar absorbtivity of reaction product, thionitrobenzoic acid ($\varepsilon_{412} = 13.600$).

Statistical analysis

Results are presented as median ± interquartile range (IQR), with a p value less than 0.05 indicating statistical significance. Differences between the groups were compared using nonparametric Kruskall Wallis test, and for the significant ($p < 0.05$) analytes Mann-Whitney's U-test was performed. All statistical calculations were made using "SPSS for Windows version 11.0" software program (SPSS Inc. Headquarters, Chicago, IL, USA).

RESULTS

Interictal period

Slightly higher levels of nitrate and nitrite in platelets of migraineurs during headache-free period were found than in control subjects but the differences were not statistically significant ($p > 0.05$) (Fig. 1). When the patients were grouped as

TABLE 2. Nitrate, nitrite, MDA and SH concentrations (μmol/10^8 cells) in migraine patients during headache-free period and during attacks.

	Control	MWOA		MWA	
		HFP	DA	HFP	DA
Nitrate (median [IQR])	4.37 (3.1)	4.63 (2.8)	6.06 (7.0)	6.35 (3.9)	9.72 (3.9)[a]
Nitrite (median [IQR])	3.98 (2.3)	5.47 (4.5)	7.71 (5.6)[a]	3.61 (4.7)	11.10 (4.8)[a,b]
MDA (median [IQR])	1.93 (1.3)	1.51 (0.8)	2.38 (4.2)[b]	2.12 (3.9)	1.78 (4.1)
SH (median [IQR])	20.73 (16.6)	19.17 (9.9)	23.10 (18.1)	28.36 (21.6)	24.35 (41.6)

Significantly higher nitrite levels were found in MWA and MWOA groups during attacks than those of control group. Nitrate levels were significantly higher in MWA group than those of controls during attack. During attacks, only MDA levels of MWOA group were significantly higher than controls. Levels of nitrate, nitrite, MDA and SH are presented as μmol in 100 million platelets.

[a] Significantly higher than control group.

[b] Significantly higher than headache-free period.

MWOA, Migraine without aura; MWA, Migraine with aura; HFP, Headache-free period; DA, During attacks; IQR, Interquartile range.

migraineurs with and without aura, platelet nitrate and nitrite levels were similarly higher in each group than controls, but were not statistically significant ($p > 0.05$) (Table 2). Platelet MDA levels were higher in migraineurs than controls but were not statistically significant ($p > 0.05$) (Fig. 2). When the patients were grouped as migraineurs with or without aura, slightly higher levels of platelet MDA were determined in each group than controls, but these were also not statistically significant ($p > 0.05$) (Table 2). There was no statistically significant difference in platelet SH concentration between patients and controls ($p > 0.05$) (Table 2).

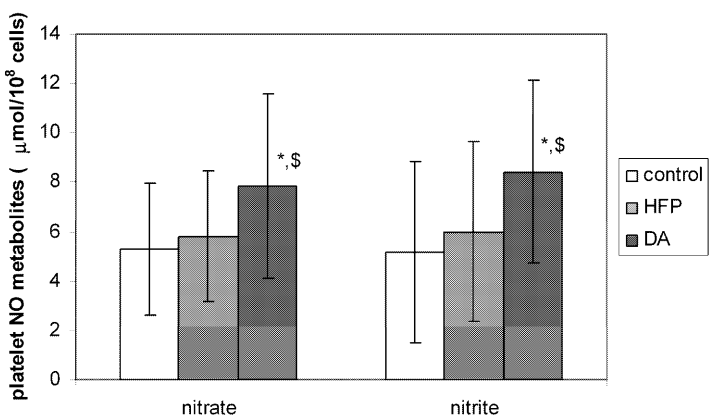

Fig. 1. Platelet nitrate and nitrite contents in migraine patients (n = 36) during headache-free period (HFP; n = 36) and during attacks (DA; n = 16) of the same patients. Samples during migraine attacks were taken from 5 patients with aura and 11 patients without aura. During HFP slightly higher levels of nitrate and nitrite in platelets of migraineurs were found than in control subjects, but the differences were not statistically significant. During attacks platelet nitrate and nitrite levels were significantly higher in migraineurs than controls (n = 20) (p = 0.042 and p = 0.005, respectively). Error bars show S.D.

*Significantly higher vs control group.

$Significantly higher vs headache free period.

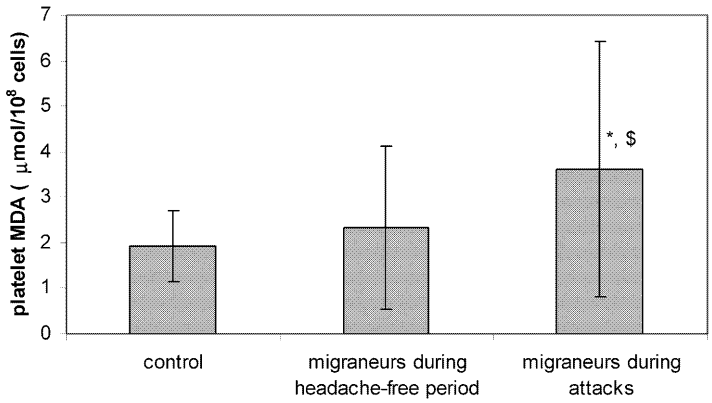

Fig. 2. Platelet MDA content in migraine patients (n = 36) during headache-free period (HFP; n = 36) and during attacks (DA; n = 16) of the same patients. Samples during migraine attacks were taken from 5 patients with aura and 11 patients without aura. During HFP platelet MDA levels were higher in migraineurs than controls (n = 20) but these were not statistically significant. At attacks MDA values were significantly greater than the values of migraineurs during headache-free period (p = 0.013). Error bars show S.D.
*Significantly higher vs control group.
$Significantly higher vs headache-free period.

Attack period

Platelet nitrate and nitrite levels were significantly higher in migraineurs than controls (p = 0.042 and p = 0.005, respectively) In the subgroups of migraine (with- and without-aura), platelet nitrate and nitrite levels were also found to be higher in each group than controls ($p < 0.05$ for both) (Table 2). Platelet MDA levels were significantly higher in migraineurs than controls (p = 0.042). When the patients were grouped as migraineurs with and without aura, significantly higher levels of platelet MDA were determined in each group than controls ($p < 0.05$ for both) (Table 2). These values were significantly greater than the values of migraineurs during headache free period (p = 0.013) (Fig. 2). There were no statistical difference in platelet SH concentrations between patients and controls ($p > 0.05$). When the patients were grouped as migraineurs with and without aura, these levels were also not different in each group than controls ($p > 0.05$) (Table 2).

Discussion

Migraine is a chronic disease with frequent attacks, high levels of pain and disability during attacks, which causes reduced quality of life between attacks (Stewart et al. 1994; Rasmussen 1995; Lipton et al. 2001; D'Amico et al. 2001; Börü et al. 2005; Gedikoglu et al. 2005; Demirkı rkan et al. 2006). It is a widespread disorder, affecting about 10-15% of general population (D'Amico et al. 2001). However, the mechanisms underlying the disease have not been clearly understood (Shukla et al. 2001). Vascular disturbance in intracranial arteries plays a significant role in the migraine attacks (Dawson et al. 1992). It was suggested that hemodynamic changes during the migraine attacks may be related to alterations in the activity of NO. NO can precipitate in the attacks by causing vasodilatation in cerebral vessels (Ciancarelli et al. 2003). It was shown that platelet hyperaggregation has an important role in migraine pathophysiology (Lechner et al. 1985; Shimomura et al. 1994). Aggregated platelets produce NO which inhibits platelet aggregation (Alheib et al. 1989; Radomski et al. 1990). Increased NO levels show a counter effect to vasoconstrictors and finally cause vasodilatation and prevention of platelet aggregation (Stepien and Chalimoniuk 1998; Thomsen 2001).

In our study, we evaluated NO activity during headache and headache-free period in migraine patients. Since NO is rapidly oxidized by tissue oxygen to the stable end products,

nitrate (NO^{3-}) and nitrite (NO^{2-}), the best index of overall NO production is the total concentration of both nitrate and nitrite (Shimomura et al. 1999). Therefore, we measured total nitrite and nitrate levels as an indicator of NO production. Similar to previous studies (D'andrea et al. 1994; Sarchielli et al. 1996; Shimomura et al. 1999), we found that platelet nitrite and nitrate levels were significantly higher in migraine patients during their migraine attack. Shimomura at al. (1999) showed that NO activity was decreased in headache-free period. In contrast to this study, we found slightly higher nitrite and nitrate levels in headache-free period of migraine patients, but this was not statistically significant. In the light of our findings, we can conclude that NO may be produced by platelets during migraine attacks and increased NO may be related to the changes in the vascular vessels and pain during migraine attacks. We think, when the NO levels exceed a critical level, the attack period may begin.

On the other hand, simultaneous release of NO and of superoxide anions produce peroxynitrite anion which is a strong biological oxidant known to oxidize lipids, proteins, and SH groups particularly (Radi et al. 1991). In the most recent study on this issue, Taffi et al. (2005) evaluated platelet peroxynitrite levels, a metabolite of nitric oxide as well as a potent oxidant, in migraine patients to resolve uncertainty about NO activity in headache-free period. They found increased peroxynitrite levels during headache-free period which are agreeable with our findings. The main reason of increased peroxynitrite concentrations is concomitant overproduction of NO and superoxide anion and these changes may result from platelet hyperaggregation. Therefore, increased nitrosative- and oxidative stress may influence platelet function and cerebral microcirculation. Platelet secretory products released by activation of lipid peroxidation may impair cerebral blood flow (Tozzi-Ciancarelli et al. 1997). It was suggested that NO may cause the headache through variations of cerebral blood flow by interacting with oxygen free radicals in migraine (Radomski et al. 1991; Shimomura et al. 1994; Ciancarelli et al. 2003). Reactive free radicals may be stimulated during migraine attacks to overcome normally effective protective mechanisms (Tozzi-Ciancarelli et al. 1997). MDA has been used to measure the degree of oxidative damage in a variety of disease (Esterbauer et al. 1991; Baldi et al. 1993; Bhatia et al. 2003). Plasma thiols which are free radical scavengers, may serve as an antioxidant in some biological processes. It was reported that the measurement of total SH levels can be used to evaluate excess free radical generation both in physiological and pathological conditions (Pasaoglu et al. 2004). Peroxynitrite induces the oxidation of platelet free SH groups in a dose-dependent way (Nowak et al. 2003). In our study, we measured platelet MDA and SH levels in migraine patients to evaluate oxidative status both during headache and headache-free period. Platelet MDA levels were higher in migraine patients during their migraine attack. However, there was no significant difference in MDA levels of migraine patients during headache free period when compared to control groups. This finding may be related to a vulnerability to oxidative stress even during headache-free period. Higher MDA levels due to increased oxidative stress in combination with increased NO activity in migraine patients may be a precipitant of a migraine attack during headache-free period. In the present study, platelet SH levels were not found to be different both in attack and attack-free period of migraine patients when compared to controls. The MDA and thiol results of the study are mutually agreeable in headache-free period. Insignificant changes in thiol groups may result from relatively strong antioxidant defence system of platelets in respect to increased peroxynitrite levels.

Study limitations

MDA assays with TBA as a biomarker for lipid peroxidation and oxidative stress have some shortcomings. Although we used homogeneous preparations of platelets, in general, TBA assays for MDA estimation in biological samples do not have a good analytical specificity. Additionally, MDA concentrations are increased with age (Özbay and Dülger 2002); and antioxidant prop-

erties of estrogen and lower concentrations of prooxidant iron may give lower MDA concentrations in premenopousal women than men (Persky et al. 2000). Although we tried hard to match the groups, age and sex variations could influence the MDA status in the present study. Since MDA is a secondary product of lipid peroxidation and needs a while to be formed from polyunsaturated fatty acids, it may not be an acutely responsive biomarker of lipid peroxidation and so it is commonly used as a chronic indicator of lipid peroxidation in vivo. Therefore, total antioxidant capacity would be a more surrogate biomarker of lipid peroxidation and oxidative stress. Additionally, the low numbers of subjects make difficulties in the interpretation of the results. Finally, although we worked hard to minimize the variation of blood collection time after the onset of attacks, a sampling period varied from 2 to 5 hrs. Relatively high variation in sampling time during attacks in patients with migraine is a common problem in such studies (Gallai et al. 1996; Shimomura et al. 1999).

In conclusion, nitrosative and oxidative stress are increased during migraine attack in platelets. Increased oxidative damaged together with increased NO activity in migraine attack may affect cerebral blood flow and cause headache. Platelet NO and MDA seem to be important molecules in migraine patients. Especially increase of NO activity in platelets during attacks, supports the opinion that NO may play a modulatory role for biological processes particularly by vasodilatation in migraine attacks. Also MDA and NO may serve as useful markers to show the increased vulnerability to nitrosative and oxidative stress in migraine patients. Further investigations with greater numbers of study subjects are necessary to clarify the exact mechanism and biological processes at the tissue level in migraine patients for the successful treatment of migraine.

References

Alheib, U., Reichwehr, I. & Forsterman, U. (1989) Human endothelial cells inhibit platelet aggregation by separately stimulating cyclic AMP and GMP. *Eur. J. Pharmacol.,* **164**, 103-110.

Baldi, E., Burra, P., Plebani, M. & Salvagnini, M. (1993) Serum malondialdhyde and mitochondrial aspartate aminotransfrease activity as markers of chronic alchol intake and alcoholic liver disease. *Ital. J. Gastreenterol.,* **25**, 429-432.

Bhatia, S., Shukla, R., Madhu, S.V., Gambhir, J.K. & Prabhu, K.M. (2003) Antioxidant status, lipid peroxidation and nitric oxide end products in patients of type 2 diabetes mellitus with nephropathy. *Clin. Biochem.,* **36**, 557-562.

Börü, Ü.T., Koçer, A., Lüleci, A., Sur, H., Tutkan, H. & Atli, H. (2005) Prevalence and characteristics of migraine in women of reproductive age in Istanbul, Turkey: A population based survey. *Tohoku J. Exp. Med.,* **206**, 51-59.

Ciancarelli, I., Tozzi-Ciancarelli, M.G., Di Massimo, C., Marini, C. & Carolei, A. (2003) Urinary nitric oxide metabolites and lipid peroxidation by-products in migraine. *Cephalalgia,* **23**, 39-42.

D'Amico, D., Mosconi, P., Genco, S., Usai, S., Prudenzano, A.M.P., Grazzi, L., Leone, M., Puca, F.M. & Bussone, G. (2001) The migraine disability assessment (MIDAS) questionnaire: translation and reliability of the Italian version. *Cephalalgia,* **21**, 947-952.

D'andrea, G., Cananzi, A.R. & Perini, F. (1994) Decreased collagen-induced platelet aggregation and increased arginin levels in migraine: a possible link with the NO pathway. *Cephalalgia,* **14**, 352-357.

Dawson, T.M., Dawson, V.L. & Synder, S.H. (1992) A novel neuronal messenger molecule in brain: free radical, nitric oxide. *Ann. Neurol.,* **32**, 297-311.

Demirkirkan, M.K., Ellidokuz, H. & Boluk, A. (2006) Prevalence and clinical characteristics of migraine in university students in Turkey. *Tohoku J. Exp. Med.,* **208**, 87-92.

Esterbauer, H., Schaur, R.J. & Zollner, H. (1991) Chemistry and biochemistry of 4 hydroxynoneal malondialdehyde and related aldehydes. *Free. Radicals Biol. Med.,* **62**, 81-128.

Gallai, V., Floridi, A., Mazzotta, G., Codini, M., Tognoloni, M., Vulcano, M.R., Sartori, M., Russo, S., Alberti, A., Michele, F. & Sarchielli, P. (1996) L-arginine/nitric oxide pathway activation in platelets of migraine patients with and without aura. *Acta Neurol. Scand.,* **94**, 151-160.

Gedikoglu, U., Coskun, O., Inan, L.E., Ucler, S., Tunc, T. & Emre, U. (2005) Validity and Reliability of Turkish Translation of Migraine Disability Assessment (MIDAS) questionnaire in patients with migraine. *Cephalalgia,* **25**, 452-456.

Headache Classification Committee of the International Headache Society (1988) Classification and diagnostic criteria for headache disorders, cranial neuralgias and facial pain. *Cephalalgia,* **8**, Suppl. 7, 1-96.

Hu, M.L. (1994) Measurement of protein thiol groups and glutathione in plasma. *Methods Enzymol.,* **223**, 380-385.

Hunter, M.I.S., Nlemadim, B.C. & Davidson, D.L.W. (1985) Lipid peroxidation products and antioxidant proteins in plasma and cerebrospinal fluids from multiple sclerosis patients. *Neurochem. Res.,* **10**, 1645-1652.

Karatas, F., Karatepe, M. & Baysar, A. (2002) Determination of free malondialdehyde in human serum by high performance liquid chromatography. *Anal. Biochem.,* **311**, 76-79.

Kitano, A., Shimomura, T., Takeshima, T. & Takahashi, K. (1994) Increased 11-dehydrothromboxane B2 in migraine: platelet hyperfunction in patients with migraine during headache-free period. *Headache,* **34**, 515-518.

Lechner, H., Ott, E., Pazekas, F. & Pilger, E. (1985) Evidence of enhanced platelet aggregation and platelet sensitivity in migraine patients. *Cephalalgia,* **5**, Suppl. 2, 89-91.

Leeksma, C.H.W. & Cohen, J.A. (1956) Determination of the

life span of human blood platelets using labelled diisopropylflourophosphonate. *J. Clin. Invest.,* **35**, 964-969.

Lipton, R.B., Stewart, W.F., Sawyer, J. & Edmeads, J.G. (2001) Clinical utility of an instrument assessing migraine disability: The migraine disability assessment (MIDAS) questionnaire. *Headache,* **41**, 854-861.

Moncada, S., Palmer, R.M.J. & Higgs, E.A. (1991) Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.,* **53**, 109-141.

Nowak, P., Olas, B., Bald, E., Glowacki, R. & Wachowicz, B. (2003) Peroxynitrite – induced changes of thiol groups in human blood platelets. *Platelets,* **14**, 375-379.

Özbay, B. & Dülger, H. (2002) Lipid peroxidation and antioxidant enzymes in Turkish population: Relation to age, gender, exercise, and smoking. *Tohoku J. Exp. Med.,* **197**, 119-124.

Pasaoglu, H., Sancak, B. & Bukan, N. (2004) Lipid peroxidation and resistance to oxidation in patients with type 2 diabetes mellitus. *Tohoku J. Exp. Med.,* **203**, 211-218.

Persky, A.M., Green, P.S., Stubley, L., Howell, C.O., Zaulyanov, L., Brazeay, G.A. & Simpkins, J.W. (2000) Protective effect of estrogens against oxidative damage to heart and skeletal muscle in vivo and in vitro. *Proc. Soc. Exp. Biol. Med.,* **223**, 59-66.

Radi, R., Beckman, J.S., Bush, K.M. & Freeman, B.A. (1991) Peroxynitrite oxydation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. *J. Biol. Chem.,* **266**, 4244-4250.

Radomski, M.W., Palmer, R.M.J. & Moncada, S. (1990) L-arginine/nitric oxide pathway present in human platelets regulates aggregation. *Proc. Natl. Acad. Sci.,* **87**, 5193-5197.

Radomski, M.W., Palmer, R.M.J. & Moncada, S. (1991) Modulation of platelet aggregation by an L-arginine-nitric oxide pathway. *Trends Pharmacol. Sci.,* **12**, 87-88.

Rasmussen, B.K. (1995) Epidemiology of headache. *Cephalalgia,* **15**, 45-68.

Sarchielli, M., Tognoloni, M., Russo, S., Vulcano, M.R., Feleppa, M., Mala, M, Sartori, M. & Gallai, V. (1996) Variations in the platelet arginine/nitric oxide pathway during the ovarian cycle in females affected by menstrual migraine. *Cephalalgia,* **16**, 468-475.

Sedlak, J. & Lindsay, R.H. (1968) Estimation of total, protein bound, and non protein sulfhydryl groups in tissue with Ellman's reagent. *Anal. Biochem.,* **25**, 192-205.

Shimomura, T., Kowa, H., Nakano, T., Kitano, A., Marukawa, H., Urakami, K. & Takahashi, K. (1994) Platelet superoxide dismutase in migraine and tension-type headache. *Cephalalgia,* **14**, 215-218.

Shimomura, T., Murakami, F., Kotaru, K., Ikawa, S. & Kono, S. (1999) Platelet NO metabolites in migraine. *Cephalalgia,* **19**, 218-222.

Shukla, R., Barthwal, M.K. & Srivastava, N. (2001) Blood nitrite levels in patients with migraine during headache free period. *Headache,* **41**, 475-481.

Smarason, A.K., Allman, K.G., Young, D. & Redman, C.W.G. (1997) Elevated levels of serum nitrate, a stable endproduct of nitric oxide, in women with preeclampsia. *Br. J. Obstet. Gynecol.,* **104**, 538-543.

Stamler, J.S., Singel, D.J. & Loscalzo, J. (1992) Biochemistry of nitric oxide and its redox activated forms. *Science,* **258**, 1898-1902.

Stepien, A. & Chalimoniuk, M. (1998) Level of nitric oxide-dependent cGMP in patients with migraine. *Cephalalgia,* **18**, 631-634.

Stewart, W.F., Shechter, A. & Lipton, R.B. (1994) Migraine heterogenity. Disability, pain intensity, and attack frequency and duration. *Neurology,* **44**, Suppl. 4, 24-39.

Taffi, R., Vignini, A., Lanciotti, C., Luconi, R., Nanetti, L., Mazzanti, L., Provinciali, L., Silvestrini, M. & Bartolini, M. (2005) Platelet membrane fluidity and peroxynitrite levels in migraine patients during headache-free periods. *Cephalalgia,* **25**, 353-358.

Thomsen, L.L. & Olesen, J. (2001) Nitric oxide in primary headache. *Curr. Opin. Neurol.,* **14**, 315-321.

Tozzi-Ciancarelli, M.G., De Matteis, G., Di Massimo, C., Marini, C., Ciancarelli, I. & Carolei, A. (1997) Oxidative stress and platelet responsiveness in migraine. *Cephalalgia,* **17**, 580-584.