The effect of vitamin E on stress-induced changes in visual evoked potentials (VEPs) in rats exposed to different experimental stress models

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ABSTRACT.
Purpose: **The aim of the study was to investigate the effects of vitamin E on stress-induced changes in visual evoked potentials (VEPs) and lipid peroxidation.**
Methods: **Eight experimental groups of 10 rats per group were formed. These consisted of the control group (C); the group treated with vitamin E (E); groups exposed to cold stress (CS), immobilization stress (IS) and both cold and immobilization stress (CIS), and groups exposed to equivalent stresses and treated with vitamin E (CSE, ISE, CISE). Vitamin E was injected intramuscularly in a dose of 30 mg/kg/day.**
Results: **Following chronic stress (15 days), plasma corticosterone concentrations in all experimental groups were significantly increased over those in C group. Vitamin E significantly decreased corticosterone levels in all stress groups compared with their respective control groups. Brain nitrite levels were significantly more elevated in all stress groups than in the C group. Vitamin E reduced retina and brain nitrite levels in all stress and E groups compared with their respective control groups. Vitamin E decreased glutathione peroxidase (GSH-Px) activity in retina and brain tissues in the CSE group, but increased it in the ISE group compared with their respective control groups. Lipid peroxidation was increased in brain and retina tissues in all stress groups as indicated by the significant increase in thiobarbituric acid-reactive substance (TBARS) levels with respect to the C group. Vitamin E produced a significant decrease in brain and retina TBARS levels in all stress groups with respect to their corresponding control groups. The mean latencies of P_1, N_1, P_2, N_2 and P_3 components were significantly prolonged in all stress groups compared with the C group.**
Conclusion: **Vitamin E returned the VEP latencies in the stress groups to control values. Our findings clearly indicated that vitamin E has the potential to prevent VEP changes caused by stress.**

Key words: vitamin E – visual evoked potentials – stress – nitric oxide – lipid peroxidation

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Introduction

Stress induces a cluster of physiological and behavioural changes (Chrousos 1998). Longterm exposure to stress has detrimental effects on several cell functions in many species, including humans (Kovacs et al. 1996). As previously shown (Kovacs et al. 1996; Liu et al. 1996; Liu & Mori 1999; Matsumoto et al. 1999; Olivenza et al. 2000), stress can stimulate numerous pathways leading to increased production of free radicals. It is well known that free radicals generate a peroxidation cascade producing lipid peroxidation, protein oxidation, DNA damage and cell death, and contribute to the occurrence of pathological conditions (Kovacs et al. 1996; Liu et al. 1996; Liu & Mori 1999). The brain and retina undergo neurodegeneration and/or atrophy because they are ideal substrates for lipid peroxidation (Matsumoto et al. 1999). In this context, previous studies have indicated that the increase of brain lipid peroxidation in response to stress was largely prevented by treatment with vitamin E (Armario et al. 1990; Askew 1995).

Vitamin E is the most prominent and well known antioxidant found in the cell membrane. Vitamin E prevents

free radical damage by chain-breaking antioxidant functions (Cheeseman & Slater 1993; Siu et al. 1999). Additionally, vitamin E, a powerful peroxyl radical remover, protects the polyunsaturated fatty acids (PUFAs) in the plasma lipoproteins and in the phospholipids of membranes (Siu et al. 1999).

The many reports indicating stress-induced lipid peroxidation lead us to expect that stress changes visual system functions. Furthermore, as neurons in the paraventricular nucleus (PVN) are activated differently under different stress models, it is possible that different stress models may have different influences on the visual system. Therefore, the present study was undertaken firstly to investigate the effects of different stress models on the visual system by means of visual evoked potentials (VEPs), which consist of several components arising from the retina, optic pathway, subcortex and cortex, because recording VEPs is known to be a highly reliable, electrophysiological method of detecting the earliest alterations in the visual system (Halliday et al. 1972; Chiappa 1983; Celesia 1984; Hudnell & Boyes 1991; Lehman & Harrison 2002). Additionally, VEP recordings from rats are good models for study of the human visual system (Hudnell et al. 1990). In the present study, immobilization, cold and immobilization + cold stress models were established to examine the relationship between increased lipid peroxidation and the changes expected to occur in the VEPs of rats exposed to these types of stress.

Consequently, in comparing the changes in lipid peroxidation in different models and using antioxidant vitamin E, an attempt has been made to clarify the importance of lipid peroxidation on possible VEP changes in stress. Thus, thiobarbituric acid-reactive substance (TBARS) was also determined as an indicator of lipid peroxidation.

Material and Methods

Preparation of animals

Healthy, male, albino rats aged 3 months were used in this study. Eight experimental groups of 10 rats per group were formed. These consisted of a control group (C); a group treated with vitamin E (E); a group exposed to cold stress (CS); a group exposed to cold stress and treated with vitamin E (CSE); a group exposed to immobilization stress (IS); a group exposed to immobilization stress and treated with vitamin E (ISE); a group exposed to both cold and immobilization stress (CIS), and a group exposed to both cold and immobilization stress and treated with vitamin E (CISE). The animals were housed in groups of 4–5 rats in stainless steel cages at standard conditions ($24 \pm 2^\circ$ and $50 \pm 5\%$ humidity) with a 12-hour light/dark cycle.

Vitamin E (30 mg/kg/day) was injected intramuscularly in the E, CSE, ISE and CISE groups.

Physiological serum was injected introperitonally in the C, CS, IS and CIS groups.

Experimental procedures

The study protocol was approved by the Akdeniz University Animal Care and Use Committee. The experiments were performed between 09 : 00 and 12 : 00. Cold and immobilization stress models were performed according to the methods described by Finlay et al. (1997) and Inoue et al. (1993), respectively.

Cold stress

The rats were placed in a cold room (ambient temperature 4–5 °) for 15 min daily for 15 days.

Immobilization stress

The rats were exposed to 180 min of immobilization stress daily for 15 days by fixing their limbs to appropriate shelves using sticky tape.

Immobilization and cold stress

Both cold and immobilization stress were applied at the same time. Animals were exposed to cold stress during the last 15 min of immobilization stress.

The daily food and water consumption of every cage and the weekly weights of each individual rat were recorded during the feeding period. The mean daily food and water consumption was estimated from the recorded values. At the end of the experimental period, rats were deprived of food for 24 hours and then prepared for the experimental procedure.

VEP RECORDINGS

Visual evoked potentials were recorded with stainless steel subdermal electrodes (Nihon Kohden NE 223 S, Tokyo Japan) under ether anaesthaesia. The reference and active electrodes were placed 0.5 cm in front of and behind the bregma, respectively. A ground electrode was placed on the tail of each animal. After 5 min of dark adaptation, a photic stimulator (Nova-Strobe AB, Biopac Systems Inc, CA) at the lowest intensity setting was used to provide the flash stimulus at a distance of 15 cm, which allowed lighting of the entire pupilla from the temporal visual field. Repetition rate of flash stimulus was 1 Hz and flash energy was 0.1 joule. Visual evoked potentials recordings from both right and left eyes were obtained, and throughout the experiments the eye not under investigation was occluded by appropriate black carbon paper and cotton. Body temperature was maintained at between 37.5 and 38°° by a heating pad (Hetzler et al. 1988).

The averaging of 100 responses was accomplished with the averager. Analysis time was 300 ms. The frequency bandwidth of the amplifier was 1–100 Hz. The gain was selected as 20 μV/div. The microprocessor was programmed to reject any sweeps contaminated with larger artifacts, and at least two averages were obtained to ensure response reproducibility.

Peak latencies of the components were measured from the stimulus artifact to the peak in milliseconds. Amplitudes were measured as the voltage between successive peaks.

Chemical analysis

After the VEP recordings, the animals were anaesthetized with diethyl ether and their abdomens were opened by midline incisions. Blood samples were taken to determine corticosterone values and the animals were killed by cardiac puncture. The brain (whole brain) and retina tissues were then removed from the rats for chemical analysis.

After a part of the tissues were removed for TBARS, nitrite and protein assays, the remaining tissues were used for the enzymatic assay of glutathione peroxidase (GSH-Px). The isolated retina was homogenized in 1.5 mL of 50 mM Tris-HCl buffer containing 1% ethanol, pH 8.5, at 0–4°°. The brain was homogenized in 4.0 mL of 5 mM phosphate buffer, pH 7.5, at 0–4 °. The homogenates were centrifuged at 20 000 ***g*** for 90 min at 0–4 °. The supernatant was employed for the enzymatic assay of GSH-Px.

Allocated tissues for nitrite, TBARS and protein assays were placed in 2 mL of ice-cold 100 mmol/L potassium phosphate buffer (pH 7.5) containing 1 mmol/L ethylenediaminetetraacetic acid (EDTA). All tissues were rapidly sonicated in a thermally regulated sonicator (Branson Sonifier 250, Schwäbisch Gmünd, Germany). Tissue sonicates were centrifuged at 14 000 *g* for 10 min at 4° in an Eppendorf microcentrifuge. The supernatant was used for the assay of TBARS, nitrite and protein.

TBARS assay

Thiobarbituric acid-reactive substance levels were measured by a fluorometric method described by Wasowicz et al. (1993) using 1, 1, 3, 3-tetramethoxypropane as a standard and the results are given as nmol/mg protein.

Nitrite assay

Nitrite levels were measured by a fluorometric method described by Ohta et al. (1986). Nitrite values are expressed as nanomoles per gram of soluble protein.

Assay of GSH-Px activity

Glutathione peroxidase activity was measured by the method described by Paglia & Valentine (1967). Data are expressed as mU/mg protein.

Corticosterone assay

In corticosterone determination Gamma-$B^{125}I$, a specific radioimmunoassay kit for rats and mice (IDS Systems Ltd, Bolden, UK, Code AA-13F_1) was used. The amount of corticosterone was calculated from the standard curve obtained from the corticosterone solutions with different concentrations in ng/mL.

Determination of protein

Protein content was determined according to Lowry et al. (1951) using bovine serum albumin as standard.

Statistical analysis

Analysis of variance (ANOVA) was performed on all parameters of VEPs for the factors of side (right and left) and groups. Differences of other data were also analysed by ANOVA. Post hoc comparisons of the means were carried out using the Tukey's *s*-test. The number of rats (*n*) was equal to 10 for each groups. Significance levels were set at $p < 0.05$.

Results

The mean plasma corticosterone levels of the studied groups are shown in Table 1. Plasma corticosterone concentrations were significantly increased in all experimental groups with respect to the C group. Vitamin E significantly decreased corticosterone levels in all stress groups compared with their respective control groups, but plasma corticosterone levels in stress groups treated with vitamin E were even higher than in their respective control groups.

The data obtained from experimental groups on nitrite and TBARS are summarized in Table 2. Brain nitrite levels were significantly elevated in all stress groups with respect to the C group. Although retina nitrite levels tended to be higher in all stress groups than in the control rats, a statistical significance was found only in the retina nitrite level of the IS group. Vitamin E reduced retina and brain nitrite levels in all stress groups compared with their respective control groups and in all experimental groups compared with the C group.

The mean GSH-Px values of the studied groups are also presented in Table 2. Glutathione peroxidase activity in brain and retina was found to be increased in the CS group, but decreased in the IS group compared with the C group. Glutathione peroxidase activity in the CIS group was also increased in the brain, but not in the retina, with respect to the C group. Vitamin E increased the retina GSH-Px value of the ISE group, but had no effect on brain GSH-Px activity in this group compared with the C group. Vitamin E reduced GSH-Px activity in the retina and brain tissues in the CSE group but increased it in the ISE group, compared with their respective control groups. Additionally, it was observed that vitamin E decreased only brain GSH-Px activity in the CISE group with respect to the CIS group.

Lipid peroxidation was measured as the amount of TBARS. As shown in Table 2, lipid peroxidation was significantly increased in retina and brain tissues in all stress groups in comparison with the C group. Vitamin E produced a significant decrease in brain and retina TBARS levels in all stress groups compared with their corresponding control groups. On the other hand, a significant decrease in lipid peroxidation was found in only the retina tissue in the E group compared with the C group.

Representative waveforms of VEPs for all groups are presented in Fig. 1. Measurements were made on three positive and two negative potentials, which were seen in all the groups.

Differences in VEP parameters were analysed by ANOVA. We did not find significant differences in latencies and amplitudes between right and left eyes. Therefore, based on the analysis, the data from stimulation of both eyes were averaged. The means and standard deviations of peak latencies and peak-to-peak amplitudes of VEP components in all groups and the results of the statistical analysis are shown in Tables 3 and 4.

The mean latencies of P_1, N_1, P_2, N_2 and P_3 components were significantly prolonged in all stress groups compared with the C group. Vitamin E significantly decreased all VEP components in stress groups compared with their respective control groups.

Table 1. Plasma corticosterone levels of studied groups.

Groups	Control (C)	Control + vitamin E (E)	Cold stress (CS)	Cold stress + vitamin E (CSE)	Immobilization stress (IS)	Immobilization stress + vitamin E (ISE)	Immobilization + cold stress (CIS)	Immobilization + cold stress + vitamin E (CISE)
Corticosterone (ng/mL)	65.44 ± 4.75	97.53 ± 2.98 C†	161.03 ± 5.14 C†	139.31 ± 7.89 C†, E†, CS*	160.38 ± 14.04 C†	118.51 ± 7.61 C†, E†, CSE* IS†	168.65 ± 4.73 C†	145.64 ± 13.53 C†, E†, ISE†, CIS*

* $p < 0.005$;
† $p < 0.001$

Table 2. Nitrite, TBARS and GSPx values of experimental groups. Values are means ± standard deviations.

Groups	Nitrite (nmol/g protein)		TBARS (nmol/g protein)		GSH−Px (mU/mg)	
	Brain	Retina	Brain	Retina	Brain	Retina
Control (C)	2.24 ± 0.18	1.68 ± 0.27	2.31 ± 0.11	2.02 ± 0.60	54.41 ± 2.92	5.04 ± 0.48
Control + vit E (E)	1.53 ± 0.48 C*	0.93 ± 0.05 C†	1.76 ± 0.70	1.03 ± 0.06 C†	56.16 ± 4.02	5.36 ± 0.10
Cold stress (CS)	2.55 ± 0.25 C†	1.82 ± 0.23	4.10 ± 0.55 C*	2.92 ± 0.16 C†	62.33 ± 2.05 C*	6.05 ± 0.15 C*
Cold stress + vit E (CSE)	1.31 ± 0.11 C*, CS*	0.81 ± 0.13 C*, CS†	2.66 ± 0.53 E†, CS*	1.52 ± 0.45 E†, CS†	57.27 ± 1.31 CS†	5.12 ± 0.61 CS†
Immobilization stress (IS)	2.64 ± 0.13 C*	1.94 ± 0.12 C†	5.59 ± 1.14 C* CS†	2.91 ± 0.38 C†	47.39 ± 3.52 C†, CS*	2.28 ± 0.06 C*, CS*
Immobilization stress + vit E (ISE)	1.45 ± 0.42 C†, IS*	0.67 ± 0.17 C*, E†, CSE†, IS*	1.79 ± 0.59 CSE†, IS*	1.31 ± 0.24 C†, IS*	52.31 ± 4.54 E†, CSE†, IS†	4.89 ± 0.43 C†, E†, IS*
Immobilization + cold stress (CIS)	2.50 ± 0.09 C*	1.83 ± 0.12	4.06 ± 0.51 C*, IS†	2.52 ± 0.14 C†, CS†, IS†	63.47 ± 2.22 C†, IS*	5.34 ± 0.67 IS†
Immobilization + cold stress + vit E (CISE)	1.55 ± 0.23 C*, CIS*	0.45 ± 0.05 C*, E*, CSE* ISE*, CIS*	2.26 ± 0.58 E†, CIS*	1.18 ± 0.23 C†, CIS*	55.33 ± 1.82 CIS*	5.17 ± 0.27 E†

* $p < 0.005$;
† $p < 0.01$

P_2N_2 amplitudes were significantly decreased in all stress groups compared with the C group. Vitamin E significantly increased P_2N_2 amplitudes in all stress groups compared with the C group.

Discussion

Plasma corticosterone levels in all stress groups were found to be significantly higher than those in the control group. Our results are in accordance with previous studies, which have demonstrated that the plasma corticosterone level is one of the important indicators of stress (Finlay et al. 1997; Kirby et al. 1997).

Our results indicated that vitamin E significantly decreased corticosterone levels in all stress groups compared with their respective control groups. Our findings are in agreement with previous studies (Ohtsuka et al. 1998; Taniguchi et al. 2001). However, vitamin E treatment had opposite effects on corticosterone in the non-stressed animals. There may be differences in the mechanism of the effects of vitamin E on corticosterone in the stressed and non-stressed animals. The reason for these contradictory results is unclear at present and further research is needed to provide the answer.

The present study provides experimental data showing that brain and retina GSH-Px activity increased in cold stress but decreased in immobilization stress compared to in the C group. Our findings are in agreement with previous reports (Ohno et al. 1991; Bondarenko et al. 1999). On the other hand, cold + immobilization stress increased only brain GSH-Px activity. Vitamin E increased retina and brain GSH-Px activity in the CSE group but decreased it in the ISE group with respect to their corresponding control groups. However, it was also observed that vitamin E decreased only brain GSH-Px activity in the CISE group compared to the CIS group. As there are no published reports on this topic, it might be concluded that the opposing GSH-Px results in different stress models could be attributed to the different mechanisms of those stress models (Pacak et al. 1995).

In agreement with previous studies (Liu et al. 1996; Bondarenko et al. 1999; Liu & Mori 1999; Matsumoto et al. 1999), our data showed that TBARS values were significantly increased during stress. One of factors for the stress-induced enhancement of lipid peroxidation may be the elevation of nitric oxide (NO) production during stress (McCann et al. 1998; Matsumoto et al. 1999). Consistent with the results of previous studies (Tsuchiya et al. 1997; Olivenza et al. 2000), nitrite levels were found to be increased in the brains of all stress groups compared with the C group. However, retina nitrite levels were significantly elevated only in rats exposed to immobilization stress. It is possible that the elevated NO may trigger a lipid peroxidation reaction in the brain and retina, probably by being converted to peroxynitrite (Chiueh 1999; Matsumoto et al. 1999; Siu et al. 1999; Olivenza et al. 2000). This idea may be supported by previous results of Shi et al. (1999), which showed that peroxynitrite can not only deplete vitamin E, but also causes production of phospholipid hydroperoxides resulting in disrupted tissues. As vitamin E has been shown to be an effective antioxidant inhibiting NO-induced lipid peroxidation in rats (Escames et al. 1997; Siu et al. 1999), it is plausible to think that depletion of vitamin E could play a role in enhancement of lipid peroxidation during stress and, in fact, corticosterone treatment was observed to increase lipid peroxidation and to decrease vitamin E in a previous study (Ohtsuka et al. 1998). This study's finding that feeding with vitamin E significantly improved these changes also supports this conclusion (Ohtsuka et al. 1998). This concept has been further supported by the present study, which revealed a significant decrease in brain and retina lipid peroxidation in stressed animals treated with vitamin E. A further striking effect of vitamin E is that it was also found to reduce corticosterone levels in stressed animals. This is

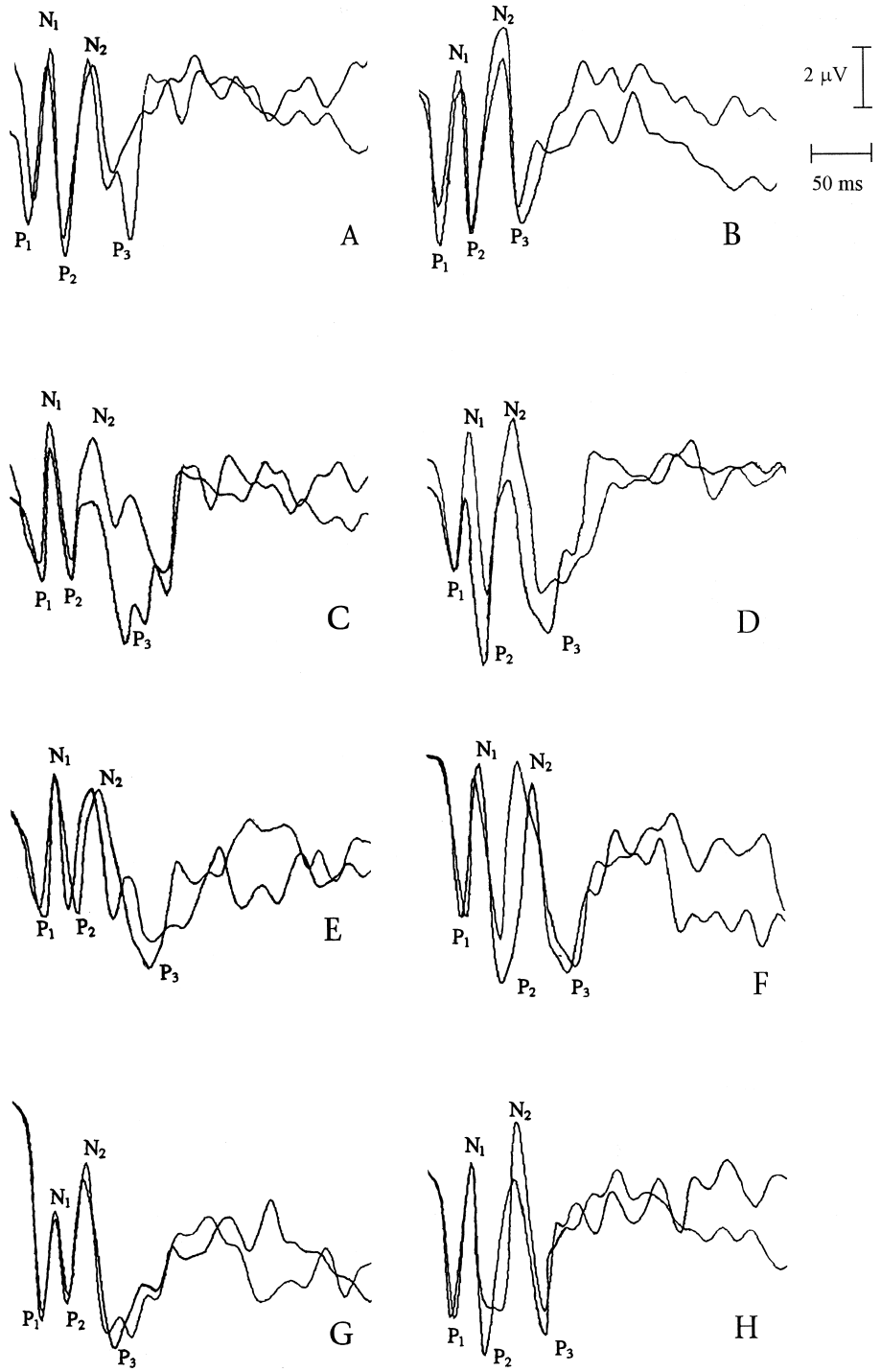


Fig. 1. Characteristic visual evoked potentials of the eight groups. Three positive (P_1, P_2, P_3) and two negative (N_1, N_2) potentials were seen in each group. (A) Control group. (B) Control + vitamin E group. (C) Cold stress. (D) Cold stress + vitamin E. (E) Immobilization stress. (F) Immobilization stress + vitamin E. (G) Immobilization + cold stress. (H) Immobilization + cold stress + vitamin E.

consistent with findings in previous studies (Cheeseman & Slater 1993; Siu et al. 1999) and this effect of vitamin E on corticosterone may be explained in the light of its antioxidant properties in preventing free radical production and lipid peroxide formation during stress. On the other hand, vitamin E decreased brain and retina nitrite levels in all experimental groups compared with their control groups. Our data are compatible with the report by Hattori et al. (1995). However, our findings contradict other data, which showed that vitamin E increased NO generation and NO synthase activity (Newaz et al. 1999). Our data also agree with previously published studies (Lathia & Blum 1989; Carr & Frei 2000) showing that addition of antioxidants dramatically inhibited NO-derived nitrosating agents such as nitrite. Therefore, it could be concluded that vitamin E has a protective effect in stress by its direct chain-breaking effect on lipid peroxidation, hence preventing the accumulation of toxic NO metabolites.

The other prominent effect of stress was observed in VEP latencies, which were prolonged in all stress groups with

Table 3. The means and standard deviations of peak latencies for each VEPs component. (The mean value of each component was determined by averaging the data for both eyes.)

Groups	P_1 (ms)	N_1 (ms)	P_2 (ms)	N_2 (ms)	P_3 (ms)
Control (C)	18.52 ± 1.53	31.21 ± 1.35	46.46 ± 2.07	70.84 ± 0.84	98.74 ± 4.26
Control + vit E (E)	19.34 ± 2.34	30.12 ± 2.78	45.34 ± 3.05	69.21 ± 1.22	101.34 ± 4.21
Cold stress (CS)	24.12 ± 2.75 C†	36.02 ± 2.38 C†	52.76 ± 2.98 C†	74.24 ± 1.52 C†	112.47 ± 2.85 C†
Cold stress + vit E (CSE)	20.05 ± 1.22 CS†	31.87 ± 3.02 CS†	45.92 ± 1.97 CS*	69.71 ± 1.27 CS†	100.42 ± 4.21 CS*
Immobilization stress (IS)	26.48 ± 0.98 C*	39.01 ± 2.42 C*, CS†	54.23 ± 1.87 C*	79.52 ± 2.63 C*, CS*	120.34 ± 3.65 C*, CS†
Immobilization stress + vit E (ISE)	19.47 ± 1.63 IS*	30.33 ± 2.21 IS*	46.21 ± 1.92 IS*	69.81 ± 2.36 IS*	99.47 ± 5.32 IS*
Immobilization + cold stress (CIS)	23.44 ± 1.32 C*, IS*	36.78 ± 1.03 C†	53.32 ± 1.21 C*	75.14 ± 2.34 C†, IS†	110.23 ± 4.23 C*, IS†
Immobilization + cold stress + vit E (CISE)	19.12 ± 1.11 CIS†	30.97 ± 1.22 CIS†	46.22 ± 2.41 CIS*	70.47 ± 1.73 CIS†	102.21 ± 3.41 CIS†

* $p < 0.005$;
† $p < 0.01$

respect to the C group. Stress-induced prolongation of VEP components probably indicates that stress markedly affects the visual system. Visual evoked potentials recording has been shown to be a sensitive and reliable method of evaluating the earliest changes in the visual system (Halliday et al. 1972; Chiappa 1983; Celesia 1984; Hudnell & Boyes 1991; Lehman & Harrison 2002). The clinical utility of VEP components for diagnosis of visual and other neurological pathologies is especially well established (Halliday et al. 1972; Chiappa 1983; Wright et al. 1986; Wright et al. 1987; Lehman & Harrison 2002). On the other hand, stress caused an increase in the lipid peroxidation process that was accompanied by changes in VEPs. Moreover, our data clearly showed that immobilization stress resulted in greater lipid peroxidation and more electrophysiological alterations than cold stress. From the results obtained, the important consequences of chronic stress on VEPs could be attributed to stress-induced lipid peroxidation. If we agree with the concept that the lipid peroxidation process may ultimately result in alterations in neural functions, we can conceivably conclude that stress-induced lipid peroxidation may play an important role in changes in VEPs parameters. Another important finding supports the statement that vitamin E returned the VEP latencies in the stress groups to control values. This finding showed that vitamin E has the potential to prevent VEP changes caused by stress.

Finally, the present study indicates that:

(1) chronic stress results in significant VEP alterations, and
(2) vitamin E may be a promising agent for the prevention of VEP alterations caused by stress-induced lipid peroxidation.

Table 4. The means and standard deviations of peak-to-peak amplitudes of each component. (The mean value of each component was determined by averaging the data for both eyes.)

Groups	P_1N_1 (μV)	N_1P_2 (μV)	P_2N_2 (μV)	N_2P_3 (μV)
Control (C)	4.25 ± 1.23	5.09 ± 2.01	7.26 ± 3.12	9.68 ± 3.12
Control + vit E (E)	5.12 ± 2.10	6.67 ± 3.78	7.67 ± 3.94	10.62 ± 2.18
Cold stress (CS)	4.73 ± 2.81	5.57 ± 2.56	4.22 ± 2.92 C*	8.21 ± 3.14
Cold stress + vit E (CSE)	5.05 ± 1.97	6.21 ± 3.87	7.23 ± 2.21 CS †	8.07 ± 4.50
Immobilization stress (IS)	5.73 ± 2.89	6.98 ± 3.12	3.83 ± 2.61 C †	6.92 ± 3.21
Immobilization stress + vit E (ISE)	5.89 ± 3.14	5.47 ± 2.12	6.21 ± 2.23 IS*	8.97 ± 4.51
Immobilization + cold stress (CIS)	5.47 ± 2.17	5.24 ± 1.89	4.02 ± 2.36 C*	7.78 ± 3.01
Immobilization + cold stress + vit E (CISE)	5.75 ± 3.74	5.21 ± 2.23	6.67 ± 2.33 CIS*	10.02 ± 3.74

* $p < 0.005$;
† $p < 0.01$

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