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Riboflavin and Vitamin E Increase Brain Calcium and Antioxidants, and Microsomal Calcium-ATP-ase Values in Rat Headache Models Induced by Glyceryl Trinitrate

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Abstract The essential use of riboflavin is the prevention of migraine headaches, although its effect on migraines is considered to be associated with the increased mitochondrial energy metabolism. Oxidative stress is also important in migraine pathophysiology. Vitamin E is a strong antioxidant in nature and its analgesic effect is not completely clear in migraines. The current study aimed to investigate the effects of glyceryl trinitrate (GTN)-sourced exogen nitric oxide (NO), in particular, and also riboflavin and/or vitamin E on involved in the headache model induced via GTN-sourced exogen NO on oxidative stress, total brain calcium levels, and microsomal membrane Ca^{2+}-ATPase levels. GTN infusion is a reliable method to provoke migraine-like headaches in experimental animals and humans. GTN resulted in a significant increase in brain cortex and microsomal lipid peroxidation levels although brain calcium, vitamin A, vitamin C, and vitamin E, and brain microsomal-reduced glutathione (GSH), glutathione peroxidase (GSH-Px), and plasma-membrane Ca^{2+}-ATP-ase values decreased through GTN. The lipid peroxidation, GSH, vitamin A, β-carotene, vitamin C, and vitamin E, and calcium concentrations, GSH-Px, and the Ca^{2+}-ATPase activities were increased both by riboflavin and vitamin E treatments. Brain calcium and vitamin A concentrations increased through riboflavin only. In conclusion, riboflavin and vitamin E had a protective effect on the GTN-induced brain injury by inhibiting free radical production, regulation of calcium-dependent processes, and supporting the antioxidant redox system. However, the effects of vitamin E on the values seem more important than in riboflavin.

Keywords Migraine · Antioxidants · Oxidative stress · Calcium · Vitamin E · Riboflavin

Abbreviations

$[Ca^{2+}]_i$	Intracellular Ca^{2+}
CSD	Cortical spreading depression
DMSO	Dimethyl sulfoxide
GSH	Reduced glutathione
GSH-Px	Glutathione peroxidase
LP	Lipid peroxidation
MMCA	Microsomal membrane Ca^{2+}-ATPase
NO	Nitric oxide
PMCA	Plasma-membrane Ca^{2+}-ATPase
ROS	Reactive oxygen species
VGCC	Voltage-gated calcium channels

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Introduction

Migraine is one of the most common neurological conditions with underlying pathophysiological mechanisms that remain unclear (Reuter et al. 2002). It has been suggested to be a neurovascular disease resulting from dysfunction of the trigeminovascular system (Yan and Dussor 2014). The blockade of presynaptic P/Q-, N-, and L-type calcium

channels present in trigeminovascular neurons inhibit the release of the calcitonin gene-related peptide and consequently the dilatation of dural blood vessels (Amrutkar et al. 2011). The clinical observations and genetic studies suggest that high-threshold voltage-gated calcium channels (VGCC) play an important role in the pathogenesis of migraine (Yan and Dussor 2014). Many cellular functions are regulated directly or indirectly via free cytosolic calcium-ion (Ca^{2+}) concentration (Nazıroğlu 2007; Kumar et al. 2014). The intracellular Ca^{2+} levels are maintained by the plasma-membrane Ca^{2+}-ATPase (PMCA) and microsomal membrane Ca^{2+}-ATPase (MMCA) in an equilibrium state. The Ca^{2+} current is considered to be associated with the release of neurotransmitters via VGCCs (Akerman et al. 2003).

It has been proven with certainty that nitric oxide (NO) plays a role as a neurotransmitter in the neurological transmission process induced by glutamate (Dawson 1995). NO inhibits mitochondrial respiration and leads to the release of glutamate by glutamate carriers, the activation of NMDA receptors and Ca^{2+} current into the cells (Brown and Bal-Price 2003). During cortical spreading depression (CSD), extracellular K^{+} increases, while levels of extracellular Na^{+} and Ca^{2+} decrease remarkably. It is considered that Ca^{2+} waves play primary role in CSD (Eikermann-Haerter and Ayata 2010). Since a typical headache, which is more common in subjects with migraines than those without migraine, develops after 4–6 h following the administration of nitroglycerin (GTN), the effect of nitroglycerin as a NO donor has been evaluated over several experimental studies (Ramachandran et al. 2014).

Oxidative stress is defined as the overproduction of reactive oxygen species (ROS) (e.g., superoxide radicals, hydrogen peroxide, and NO) (Nazıroğlu 2007; Paredes and Reiter 2010). The generation of ROS is ubiquitous since ROS are generated during aerobic metabolism, such as mitochondrial oxidations and other monoamine oxidants (Daiber et al. 2013). The results of recent studies indicated that oxidative stress is the main source in the etiology of migraines (Yilmaz et al. 2011; Ishii et al. 2011). In order to scavenge, various ROS defense systems exist in the brain. Glutathione peroxidase (GSH-Px) is responsible for the reduction of hydro and organic peroxides in the presence of reduced glutathione (GSH) (Nazıroğlu and Yürekli 2013; Yürekli and Nazıroğlu 2013). GSH is the most abundant thiol antioxidant in mammalian cells and maintains thiol redox in the cells. GSH depletion has been implicated in the neurobiology of neurons (Nazıroğlu et al. 2011). Vitamin E, alpha-tocopherol, is the most important antioxidant in the lipid phase of cells and it modulates also Ca^{2+} entry in neurons (Nazıroğlu and Özgül 2013). Vitamin C, as well as being a free radical scavenger, also transforms vitamin E to its active form. As such, the positive effect of vitamin E is well-known on systemic diseases such as diabetes and cardiovascular diseases and neurological diseases such as epilepsy and depression although its effects on migraine prophylaxis is not clearly determined.

Riboflavin is an agent used in the prophylactic treatment of migraine. It has been reported in preclinical studies that riboflavin may reduce pain in mice (França et al. 2001). Riboflavin is a water-soluble vitamin that works as a coenzyme in the mitochondrial electron transport chain (Colombo et al. 2014). Mitochondria are one of main sources of ROS in cells, although there are no reports on oxidative stress and the subject of riboflavin on migraines. It is considered that it exhibits its positive functional effects by increasing the activities of complexes I and II and also mitochondrial energy metabolism (Markley 2012). However, this issue is still controversial (Colombo et al. 2014).

It has not been studied whether vitamin E and riboflavin modify the alterations in the antioxidant enzyme system and lipid peroxidation levels in the brain and microsomal in rats. Hence, we aimed to evaluate whether there would be a protective effect of vitamin E and riboflavin on oxidative stress and enzymatic antioxidants, MMCA, and calcium values in GTN-induced brain injury in experimental migraine rat models.

Materials and Methods

Animals

The current study included 60 female Wistar-Albino rats aged 8–12 weeks and weighing 200–250 g. The approval was obtained from the Ethics Committee of the Medical Faculty of Suleyman Demirel University to use experimental animals for scientific purposes. All of the rats were held under standard conditions of light (12 h of daylight/12 h of darkness) and heat (~25 °C). All of the rats were given enough water and standard rat food pellets and put into separate chambers of the cage.

Experimental Design and Induction of Migraine

The rats were distributed randomly into four groups including 15 rats in each group. The groups were determined as Group 1: Control Group; Group 2: Glyceryl trinitrate (GTN) Group; Group 3: GTN + RBF (riboflavin) Group; Group 4: Riboflavin + GTN + vitamin E Group.

Control Group

Rats in Group 1 received a placebo over 10 days of the study.

GTN Group

Animals in the groups received a placebo over 10 days of the study and then GTN was administered to the animals.

Riboflavin + GTN Group

Rats in Group 3 received 100 mg/kg/day oral riboflavin (Eczacibasi Pharmaceuticals Corporation, Istanbul, Turkey) (Granados-Soto et al. 2004) for 10 days before GTN administration.

Riboflavin + GTN + Vitamin E Group

Rats in the Group 4 received oral riboflavin (100 mg/kg/day) plus 100 mg/kg/vitamin E (Evigen, Eras Inc., Istanbul, Turkey) intraperitoneally every other day (i.p.) for 10 days before GTN administration (Nazıroğlu et al. 2004).

We used the vitamin E and riboflavin in pretreatment groups for 10 days before GTN administrations. Twenty-four hours following the last medicinal treatment, an experimental headache model was induced with a three-hour-infusion of 10 mg/kg i.p. GTN to Groups 2, 3, and 4 (Ramachandran et al. 2012). Group 1 was established as the control group via administrating the same amount of physiological serum i.p. One hour after GTN infusion, rats showed decelerated motion and decreased reaction to pulling test of rat-tail (Ramachandran et al. 2012). GTN (Sigma, Istanbul, Turkey), dissolved in saline, alcohol, and propylene glycol was injected i.p. at a dose of 10 mg/kg.

Anesthesia and Preparation of Brain Samples

The animals, whose feeding was interrupted the previous night, were administered mixture of ketamine hydrochloride (50 mg/kg) and xylazine (5 mg/kg) i.p. 1 h after the 3-h GTN infusion. Then, all of the rats were sacrificed and their brain cortexes were removed. The removed tissues of brain cortex samples were washed twice with cold physiological saline. They were held in glass bottles in a deep freeze (−30 °C) for a specified period (maximum 10 h). Next, cortex samples were minced on the ice. They was homogenized at 5,000 rpm for two minutes using cold-ice Tris–HCl buffer (50 mM, pH 7.4) in a Teflon homogenizer. Microsomes were isolated from the remaining samples of the brain cortex. All of the samples were stored at +4 °C.

Isolation of Brain Microsomes

Brain samples were cleaned and minced and then homogenized in six volumes of freshly prepared buffer A containing: 0.3 mol/l sucrose, 10 mmol/l Hepes- HCl buffer pH 7.4, and 2 mmol/l dithiothreitol. The material was homogenized with the glass Teflon homogenizer. The homogenate was centrifuged (MS 80, Sanyo Inc.) at 85,000×*g* (Sorvall, Teknolab A.Ş, Ankara, Turkey) for 75 min. The supernatant was discarded and the pellet was re-suspended in the original volume of buffer A containing 0.6 mol/l KCl using four strokes of the pestle and centrifuged again at 85,000×*g* for 75 min. The pellets were re-suspended in the original volume of buffer A. After centrifugation at 85,000×*g* for 75 min, the pellet was suspended in buffer A using four strokes of the pestle to produce a protein concentration of 2–7 mg/ml. The complete procedure took 10–12 h for eight samples. The samples were frozen and stored at −33 °C until assayed. The isolation procedure was carried out at +4 °C (Nazıroğlu et al. 2009; Calişkan et al. 2010).

Measurement of Microsomal Ca^{2+}-ATPase Activity

The Ca^{2+}-ATPase activity was measured spectrophotometrically using the method of Burette et al. (2003). The assay medium contained: 120 mmol/l KCl, 60 mmol/l Hepes buffer, pH 7 (at 37 °C), 1 mmol/l $MgCl_2$, 0.5 mmol/l K_2-ATP, 0.2 mmol/l NADH, 0.5 mmol/PEPA, 1 IU/l pyruvate kinase, 1 IU/l LDH, and 500 mmol/l EGTA. After pre-incubation of the assay medium (total volume of 1 ml) for 4 min at 37 °C, 50 mg of the microsomal homogenate was added to the medium. After 2 min, the reaction was initiated by the addition of $CaCl_2$ (600 mmol/l). The ATPase activity as oxidation of NADH was followed by continuously measuring the absorbance at 340 nm.

Based on the extinction coefficient for NADH, $\varepsilon = 6.2 \times 10^6\ M^{-1}$, the amount of NADH oxidized was equivalent to the hydrolyzed amount of ATP. Values were expressed as IU/mg protein.

Measurement of Total Brain Calcium Concentrations

Microsomal calcium levels were analyzed with a plasma optic emission atomic absorption spectrophotometer (ICP-OES, Optima 4300 DV, Perkin Elmer Life and Analytical Sciences, Inc. Waltham, Massachusetts, USA) by following the wet ashing procedure with nitric acid (Nazıroğlu et al. 2008). Ca values were measured at 422.7 nm in the atomic absorption spectrophotometer.

Lipid Peroxidation Level Determinations

Lipid peroxidation levels in the brain homogenate and microsomal samples were measured with the thiobarbituric-acid reaction by the method of Placer et al. (1966). The quantification of thiobarbituric-acid reactive substances was determined by comparing the absorption to the

standard curve for malondialdehyde (MDA) equivalents generated by acid-catalyzed hydrolysis of 1,1,3,3-tetramethoxypropane. The values of lipid peroxidation in the brain and microsomal were expressed as μmol/g protein.

Brain Cortex and Microsomal-Reduced Glutathione (GSH), Glutathione Peroxidase (GSH-Px) and Protein Assay

The GSH contents of the brain homogenate and microsome were measured at 412 nm using the method of Sedlak and Lindsay (1968) as described in the previous studies (Nazıroğlu et al. 2004, 2008). GSH-Px activities of the brain homogenate and microsome were measured spectrophotometrically at 37 °C and 412 nm according to Lawrence and Burk (1976). The protein content in the brain cortex and microsomes was measured by method of Lowry et al. (1951) with bovine serum albumin as the standard.

Brain β-carotene, Vitamins A, C, and E Analyses

Vitamins A (retinol) and E (α-tocopherol) were determined in the brain samples by a modification of the method described by Desai (1984) and Suzuki and Katoh (1990). Brain samples of approximately 0.25 g were saponified by the addition of 0.3 ml 60 percent (w/v in water) KOH and 2 ml of one percent (w/v in ethanol) ascorbic acid, followed by heating at 70 °C for 30 min. After cooling the samples on ice, 2 ml of water and 1 ml of *n*-hexane were added and mixed with the samples and then allowed to rest for 10 min to allow for phase separation. An aliquot of 0.5 ml of *n*-hexane extract was taken and vitamin A concentrations were measured at 325 nm. Then reactants were added and the absorbance value of hexane was measured in a spectrophotometer at 535 nm. Calibration was performed using standard solutions of all-trans retinol and α-tocopherol in hexane.

The concentrations of β-carotene in brain samples were determined according to the method of Suzuki and Katoh (1990). Two ml of hexane were mixed with 0.25 g brain. The concentration of β-carotene in hexane was measured at 453 nm in a spectrophotometer.

Statistical Analyses

The statistical analyses were performed using the pocket software of *SPSS 15.0 for Windows*. The Mann–Whitney *U*-test was used for the comparison between the groups with respect to Ca^{+2}-ATPase and total brain calcium concentration. The results were expressed as mean value ± SD. $p < 0.05$ was accepted as significant.

Results

Results of Brain Microsomal Ca^{2+}-ATPase Activities

The effects of riboflavin and vitamin E on Ca^{2+}-ATPase activity in brain of headache-induced rats are shown in Fig. 1. The mean brain microsome Ca^{2+}-ATPase activities as IU/mg protein in control, GTN and riboflavin + GTN and riboflavin + vitamin E + GTN groups were 0.14, 0.06, 0.09, and 0.12, respectively. The Ca^{2+}-ATPase activity was significantly lower in the GTN ($p < 0.001$) and riboflavin + GTN ($p < 0.05$) groups compared to the control. However, the Ca^{2+}-ATPase activity was significantly higher in the riboflavin + GTN ($p < 0.05$) and riboflavin + vitamin E + GTN ($p < 0.001$) groups compared to the GTN group. Hence, we observed the protective effects of riboflavin and vitamin E on brain Ca^{2+}-ATPase activity in the GTN-induced toxicity of rats. However, it seems that the protective effects of riboflavin + vitamin E on Ca^{2+}-ATPase activity was significantly higher than in the riboflavin treatment group in the brain samples.

Results of Brain Calcium Concentration

The effects of riboflavin and vitamin E on the brain calcium concentration in the brains of migraine-induced rats are shown in Fig. 2. The mean brain calcium concentrations as mg/g wet tissue in the control, GTN and riboflavin + GTN and GTN + riboflavin + vitamin E groups were 5.99, 3.73, 4.09, and 6.66, respectively. The brain calcium concentration was significantly ($p < 0.001$) lower in the GTN and riboflavin + GTN groups compared to the control. However, the brain calcium concentration was significantly ($p < 0.001$) higher in the riboflavin + vitamin E + GTN group compared to the GTN and riboflavin + GTN groups. Hence, we observed the protective effects of vitamin E on brain calcium concentration in GTN-induced toxicity of rats. However, it seems that there were no protective effects of riboflavin on calcium concentrations in the brain samples.

Results of Brain Lipid Peroxidation, GSH, and GSH-Px Values

Lipid peroxidation, GSH, and GSH-Px results are indicated in Table 1. The results indicated that lipid peroxidation levels were markedly ($p < 0.05$) higher in the GTN group compared to the control group, although GSH levels were markedly ($p < 0.05$) lower in the GTN group compared to the control. However, the lipid peroxidation levels decreased in the riboflavin + GTN and riboflavin + vitamin E + GTN administrated groups compared to the GTN

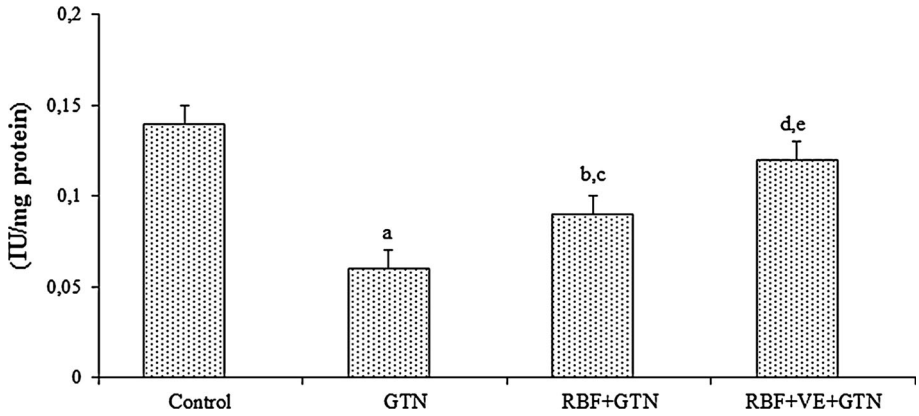

Fig. 1 Effects of riboflavin (RBF) and vitamin E (VE) on Ca^{2+}-ATPase activity in the brain microsomes of migraine-induced rats ($n = 15$ and mean ± SD). [a]$p < 0.001$ and [b]$p < 0.05$ versus the control. [c]$p < 0.05$ and [d]$p < 0.001$ versus the GTN group. [e]$p < 0.05$ versus the GTN + RBF group

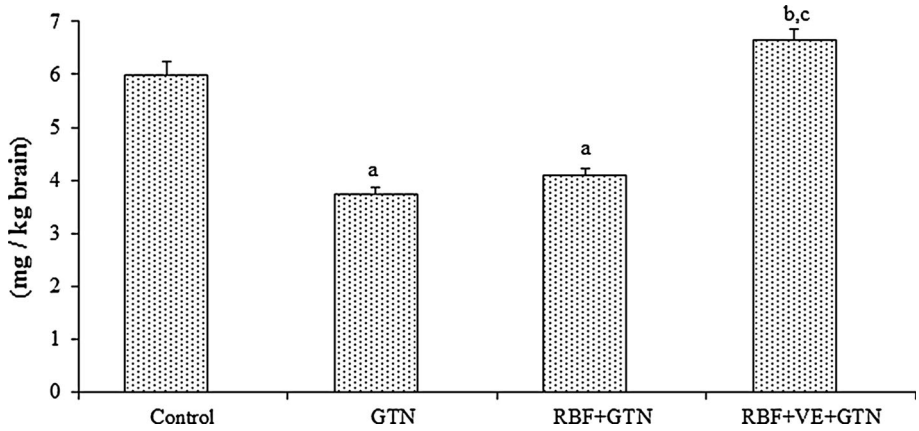

Fig. 2 The effects of riboflavin (RBF) and vitamin E (VE) on brain calcium concentration in the brains of migraine-induced rats ($n = 15$ and mean ± SD). [a]$p < 0.001$ versus the control. [b]$p < 0.001$ versus the GTN group. [c]$p < 0.001$ versus the GTN + RBF group

Table 1 The effects of riboflavin (RBF) and vitamin E (VE) on brain lipid peroxidation (LP), glutathione peroxidase (GSH-Px) and reduced glutathione (GSH) values in GTN-induced brain injury in rats (mean ± SD)

Parameters	Control ($n = 15$)	GTN ($n = 15$)	RBF + GTN ($n = 15$)	RBF + VE + GTN ($n = 15$)
GSH-Px (IU/g protein)	27.49 ± 4.22	26.00 ± 2.99	28.94 ± 4.38	27.67 ± 4.38
GSH (μmol/g protein)	10.90 ± 1.78	9.48 ± 1.08*	11.40 ± 1.32[a]	11.46 ± 1.04[a]
LP (μmol/g protein)	13.39 ± 2.09	14.14 ± 3.11*	12.32 ± 1.48[a]	11.51 ± 1.48[a]

* $p < 0.05$ versus the control

[a] $p < 0.05$ versus the GTN group

group ($p < 0.05$) only. There was no statistical change in GSH-Px activity in the four groups.

Results of Brain Microsome Lipid Peroxidation, GSH, and GSH-Px Values

Lipid peroxidation, GSH, and GSH-Px value results of the brain microsome in the four groups are shown in Table 2. The results indicated that lipid peroxidation levels were markedly ($p < 0.05$) higher in the GTN group compared to the control group, although GSH-Px activity and GSH levels were markedly ($p < 0.05$) lower in the GTN group compared to the control group. However, the lipid peroxidation levels were significantly lower in the riboflavin + GTN ($p < 0.05$) and riboflavin + vitamin E + GTN ($p < 0.05$) groups compared to the GTN group alone.

Results of Brain Antioxidant Vitamin Concentrations

Vitamin A, vitamin E, vitamin C, and β-carotene concentrations in the total brain in the four groups are shown in Table 3. Vitamin A ($p < 0.05$), vitamin C ($p < 0.05$), and vitamin E ($p < 0.01$) concentrations were markedly lower in the GTN group compared to the control. However, the

Table 2 The effects of riboflavin (RBF) and vitamin E (VE) on brain microsomal lipid peroxidation (LP), glutathione peroxidase (GSH-Px), and reduced glutathione (GSH) values in GTN-induced brain injury in rats (mean ± SD)

Parameters	Control ($n = 15$)	GTN ($n = 15$)	RBF + GTN ($n = 15$)	RBF + VE + GTN ($n = 15$)
GSH-Px (IU/g protein)	16.28 ± 2.56	14.05 ± 4.33*	15.26 ± 4.09[a]	15.52 ± 4.09[a]
GSH (μmol/g protein)	7.20 ± 1.98	7.54 ± 1.74*	12.00 ± 2.86[a]	10.38 ± 2.04[a]
LP (μmol/g protein)	21.29 ± 1.88	24.65 ± 2.72*	20.49 ± 1.96	18.35 ± 1.96[b]

* $p < 0.05$ versus the control

[a] $p < 0.05$ and [b] $p < 0.01$ versus the GTN group

β-carotene, vitamin C, and vitamin E concentrations were significantly ($p < 0.05$) higher in the riboflavin + GTN group compared to the GTN group. The vitamin A ($p < 0.05$), β-carotene ($p < 0.01$), vitamin C ($p < 0.01$), and vitamin E ($p < 0.05$) concentrations were also significantly higher in the riboflavin + vitamin E + GTN group compared to the GTN group. The effects of vitamin E on the vitamin concentrations in the brain samples seem most significant compared to the riboflavin group.

Discussion

We observed that the brain and microsomal lipid peroxidation levels were increased by GTN administration, although brain and microsomal GSH, brain calcium, vitamin A, vitamin C, and vitamin E concentrations, and MMCA activities decreased. Hence, GTN administrations in animals are characterized by increased oxidative stress and decreased MMCA, calcium, GSH, and antioxidant vitamin values. The administration of riboflavin and vitamin E induced a decrease in calcium and brain and microsomal lipid peroxidation levels, although GSH, vitamin A, β-carotene, vitamin E, and vitamin C concentrations and MMCA activity increased. A limited number of in vivo or in vitro studies in the brain of experimental animals have been reported regarding the effects of riboflavin and vitamin E on antioxidant enzymatic system, lipid peroxidation, and MMCA values (Hassan et al. 2013; Das Evcimen et al. 2004). To the best of our knowledge, the current study is the first to compare riboflavin and vitamin with particular reference to their effects on oxidative stress and the antioxidant redox system in GTN-induced brain injury in a migraine rat model.

Inactivation of ROS can be conducted by antioxidant vitamins (Nazıroğlu 2007b). Vitamin E (α-tocopherol) is the most important antioxidant in the lipid phase of cells. Vitamin E acts to protect cells against the effects of ROS, which are potentially damaging byproducts of the body's metabolism (Nazıroğlu et al. 2004; Nazıroğlu and Özgül 2013). Vitamin C and GSH, in addition to being two free radical scavengers, also transform vitamin E to its active form (Nazıroğlu 2007; Daiber et al. 2013). It is well known that CSD induces oxidative stress in the brain and trigeminal nociceptive system (Shatillo et al. 2013). Riboflavin is a well-known nutritional supplement that has been shown to exhibit antioxidant properties and protect the brain from oxidative damage (Hassan et al. 2013). The antioxidant levels in the brain were considerably low. Therefore, low antioxidant levels and high content of PUFA result in limited antioxidant defense in the brain. Vitamin A, vitamin C, and vitamin E concentrations in the brain cortex decreased in the GTN group, although their concentrations in the brain cortex increased in the riboflavin and vitamin E treatment groups. The increased

Table 3 The effects of riboflavin (RBF) and vitamin E (VE) on antioxidant vitamin values in GTN-induced brain injury in rats (mean ± SD)

Parameters	Control ($n = 15$)	GTN ($n = 15$)	RBF + GTN ($n = 15$)	RBF + VE + GTN ($n = 15$)
Vitamin A (μmol/g brain)	3.22 ± 0.56	2.78 ± 0.46*	2.89 ± 0.10	3.24 ± 0.53[a,c]
β-carotene (μmol/g brain)	2.37 ± 0.28	2.27 ± 0.38	2.48 ± 0.12[a]	2.61 ± 0.14[b,a]
Vitamin C (μmol/g brain)	1.31 ± 0.15	1.04 ± 0.27*	1.25 ± 0.25[a]	2.00 ± 0.38[b,d]
Vitamin E (μmol/g brain)	12.11 ± 1.51	7.10 ± 1.34**	8.20 ± 1.16[a]	10.64 ± 1.24[b,d]

* $p < 0.05$ and ** $p < 0.01$ versus the control

[a] $p < 0.05$ and [b] $p < 0.01$ versus the GTN group

[c] $p < 0.05$ and [d] $p < 0.01$ versus the GTN + RBF group

concentrations of the antioxidant vitamins could be due to its depletion or inhibition as a result of the increased production of free radicals. The increase in the brain cortex vitamin A, vitamin C, and vitamin E values in animals during riboflavin and vitamin E treatments has been attributed to the inhibition of free radicals and lipid peroxidation (Dyatlov et al. 1998; Erol et al. 2010; Özkaya et al. 2011; Ceylan et al. 2011; Sanches et al. 2014).

The selenium-dependent GSH-Px antioxidant enzyme is responsible for the reduction of hydro and organic peroxides in the presence of GSH (Nazıroğlu and Yürekli 2013; Yürekli and Nazıroğlu 2013). GSH is the most abundant thiol antioxidant in mammalian cells and maintains thiol redox in the cells. GSH depletion has been implicated in the neurobiology of neurons (Nazıroğlu et al. 2011). The results of recent papers indicated that vitamin E (Özkaya et al. 2011; Ceylan et al. 2011) and riboflavin (Sanches et al. 2014) supplementation induced an increase of GSH and GSH-Px values in animal and human studies due to the modulation of mitochondrial ROS production process (Siler-Marsiglio et al. 2005; Xie et al. 2013). In the current study, we observed brain GSH, brain and microsomal GSH, and GSH-Px values were low in the GTN group, although they increased with vitamin E and riboflavin supplementation and the effects of vitamin E and riboflavin might be induced by the modulation of the mitochondrial ROS production process. To our knowledge, there have been no reports on the effects of vitamin E and riboflavin in GTN-induced brain injury in human and experimental animals. However, there are a few reports on the antioxidant enzyme values in patients with migraines. Erol et al. (2010) and Vurucu et al. (2013) reported that GSH-Px activity was low in adult and pediatric patients with migraines. Hence, GSH-Px results of the current study were supported by the results of Erol et al. (2010) and Vurucu et al. (2013).

Mitochondria were reported to accumulate Ca^{2+} provided cytosolic Ca^{2+} rises or provided mitochondrial uptake dominates mitochondrial Ca^{2+} extrusion, thereby leading to the depolarization of mitochondrial membranes (Kovács et al. 2002; Espino et al. 2010). The uptake of Ca^{2+} into mitochondria stimulates the tricarboxylate cycle, resulting in an augmented reduction of pyridine nucleotides, which may be one of the mechanisms of the coupling of neuronal and metabolic activity (Kumar et al. 2014). On the other hand, the exposure of mitochondria to high cytosolic free Ca^{2+} was shown to increase the formation of ROS (Nazıroğlu et al. 2014). It has been reported that vitamin E and riboflavin induced the modulator role on ROS production and mitochondrial functions (Siler-Marsiglio et al. 2005; Xie et al. 2013) and also reduced cytosolic Ca^{2+} levels by the regulation of VGCC and TRP cation channels (Huang et al. 2000; Nazıroğlu and Özgül 2013). In the current study, the brain cortex and microsomal lipid peroxidation and MMCA values were lower in the riboflavin and vitamin E groups compared to the GTN group. Modulation of VGCC and TRP channels in the brain cells by means of the treatment with riboflavin and vitamin E might has caused a decrease in mitochondrial ROS productions and Ca^{2+} entry.

The fluctuations in the levels of intracellular calcium levels affect the physiological, chemical, and biological processes (Kumar et al. 2014). CSD is the transient suppression of neuronal activity resulting from the temporary disruption of local brain ionic homeostasis (Eikermann-Haerter and Ayata 2010). The production of NO and entry of Ca^{2+} in cytosol increase during CSD (Dawson 1995; Akerman et al. 2003). NO inhibits mitochondrial respiration and leads to the release of glutamate via glutamate carriers (Eikermann-Haerter and Ayata 2010). Exogen NO facilitates CSD that occurs due to excitatory neurotransmitters (Dawson 1995). Similar to CSD, extracellular calcium decreased 1 mmol (Jing et al. 1993), while intracellular calcium increased less than 0.2 μmol (Wang et al. 2001) at 37 °C along hypoxic spreading depression. Therefore, reduction in the extracellular calcium levels are higher than the elevation in the intracellular calcium levels along CSD. In this case, the reduction in total calcium levels along CSD is possible. The results of our study support this hypothesis. In our study, total brain calcium levels significantly decreased when compared to the control group in the rats in which an experimental headache model was induced via GTN-sourced exogen NO.

These results caused us consider that riboflavin and/or vitamin E improved the disrupted calcium-ion balance in the rats in which headache was induced by GTN. Riboflavin inhibits guanylate cyclase (Galagan et al. 1991) and the inhibition of guanylate cyclase decreases the presynaptic release of NO and consequently reduces the NMDA-mediated calcium current into the cell. Finally, it was demonstrated that riboflavin inhibits the release of glutamate from the cerebrocortical nerve ends at a significant ratio and suppress VGCCs. In the current study, total brain calcium levels significantly increased in the group in which riboflavin was treated compared to the GTN group. The findings obtained in the current study may be interpreted in favor of the inhibition of guanylate cyclase or the suppression of VGCC by riboflavin. However, the effects and the action mechanism of riboflavin over brain calcium homeostasis in migraines is not completely clear yet. In this study, significantly increased total brain calcium levels were observed in the riboflavin + vitamin E group when compared with the riboflavin group. NO, as a free radical, increased the activation of NMDA receptors. Vitamin E may affect the NMDA-mediated calcium current; however, various findings have been reported in the studies on this issue.

Nazıroğlu and Luckhoff (2008) and Nazıroğlu and Özgül (2013) reported that vitamin E, well-known for its membrane stabilizing effect, decreases the permeability of cell membrane against various ions including Ca^{2+}; whereas, Dyatlov et al. (1998) demonstrated that α-tocopherol increased the Ca^{2+} current significantly in the cells with low energy. The findings obtained in the current study also supported these results, in which Vitamin E increased total brain calcium levels.

In conclusion, the current study's brain results in the GTN group are consistent with a generalized antioxidant abnormality in different tissues of animals and humans with migraines and headaches. However, riboflavin and vitamin E supplementation induced a protective effect on the oxidative stress and antioxidant redox system in the brain cortex and microsomes. The beneficial effect of riboflavin and vitamin E on enzymatic antioxidant systems was the regulation of GSH, antioxidant vitamins, MMCA activities, and lipid peroxidation levels in the brain. Hence, use of the riboflavin with vitamin E could be a potential approach in arresting or inhibiting the headache genesis caused by excitotoxic agents.

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