Klinik Çalışma - Araştırma / *Original Article*

Effects Of Darbepoetin Alpha on Brain Tissue Oxidative Stress in Experimental Ethanol Administration

Deneysel Etanol Uygulamasında Darbepoetin Alfanın Beyin Dokusu Oksidatif Stresi Üzerine Etkileri

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Objectives: The hyperglycosylated erythropoietin analogue darbepoetin alpha (α) has longer half-life and higher in vivo activity. There is no data about the effects of darbepoetin-α on ethanol-induced oxidative stress. In this study, we investigated the effects of darbepoetin-α on brain tissue oxidant/antioxidant status and nitric oxide levels in experimental ethanol administration.

Patients and Methods: Forty-four adult male Wistar albino rats were randomly divided into groups: saline-treated group (S) (n=10), saline and darbepoetin-treated group (D) (10 μg/kg) (n=10), experimental ethanol-administered [2.5 g/kg (2.6 ml/kg) twice at 2-hr intervals] group (E) (n=12), ethanol-administered and darbepoetin-treated group (ED) (n=12).

Results: Malondialdehyde (MDA) levels of ED group were significantly lower than E group ($p<0.05$). Glutathione (GSH) levels of ED group were significantly higher than E group ($p<0.001$). NO levels of ED group were significantly lower than E group ($p<0.001$).

Conclusion: We have observed that darbepoetin-α decreases oxidants and increases antioxidants against ethanol-induced oxidative stress. Darbepoetin-α is protective in ethanol-induced organism via its antioxidant activity.

Key words: Darbepoetin alpha; oxidative stress; NO; lipid peroxidation.

Amaç: Eritropoetin analoğu darbepoetin alfa, yarı ömrü uzun ve in vivo yüksek aktiviteye sahip bir moleküldür. Alkolün etkili olduğu organizmada darbepoetinin etkisi henüz ortaya konulmamıştır. Bu çalışmada, deneysel etanol uygulamasında darbepoetin alfanın beyin dokusu oksidan/antioksidan denge ve nitrik oksit seviyesi üzerine etkilerini araştırdık.

Hastalar ve Yöntemler: Kırk dört erişkin erkek Wistar Albino sıçan raslantısal olarak gruplara ayrıldı: %0.9 NaCl uygulanan grup (S) (n=10), %0.9 NaCl ve darbepoetin verilen grup (D) (10 μg /kg) (n=10), deneysel etanol uygulaması yapılan [2.5 g/kg (2.6 ml/kg) 2-saatlik aralıklar ile iki defa] grup (E) (n=12), etanol uygulanan ve darbepoetin verilen grup (ED) (n=12).

Bulgular: ED grubu malondialdehit (MDA) değerleri E grubundan anlamlı olarak düşüktü ($p<0.05$). ED grubu glutatyon (GSH) değeri E grubundan anlamlı olarak yüksekti ($p<0.001$). ED grubu NO değerleri E grubundan anlamlı olarak düşüktü ($p<0.001$).

Sonuç: Deneysel etanol uygulaması modelinde darbepoetin alfanın oksidanları düşürdüğünü, antioksidanları ise artırdığını gözlemledik. Darbepoetin alfa antioksidan aktivitesi ile alkol uygulanan organizmada koruyucu etkilere sahiptir.

Anahtar sözcükler: Darbepoetin alfa; oksidatif stres; NO; lipit peroksidasyonu.

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Alcohol consumption is a common habit in many countries. However, ethanol and its oxidation products cause oxidative damage and toxicity in various organs such as liver and brain.[1-3] The generation of lipid peroxidation by free radicals has been proposed as a mechanism for ethanol induced toxicity. Acetaldehyde is a cytotoxic product of ethanol metabolism. Acetaldehyde is further oxidized to acetate by acetaldehyde dehydrogenase enzyme which is present in the brain and is capable of producing reactive oxygen species (ROS).[1] Acetaldehyde is able to react with protein structures to form adducts which can interact with proteins and fatty acids to cause many adverse metabolic effects.[4] Ethanol increases the NADH/NAD ratio, which causes reduction of ferric iron to ferrous iron, a potent generator of the hydroxyl radical, which is suggested as the cause of lipid peroxidation leading to loss of membrane integrity.[1,2]

Recently the neuroprotective and ameliorating effect of recombinant human erythropoietin (rHu-EPO) in ethanol-induced neuronal cell death have been shown.[1] Additionally, beneficial effects of erythropoetin have been reported in injured rat brain against oxidative stress.[5] The hyperglycosylated rHuEPO analogue darbepoetin-alpha (α) has higher sialic acid content compared to rHuEPO, resulting in longer (3-4 times) half-life and higher invivo activity.[5-7] Although darbepoetin-α is commonly used for correction of anemia in patients with chronic renal disease, it is postulated that darbepoetin-α has neuroprotective effects like that of rHuEPO because it binds the same receptor with rHuEPO.[8] Likewise, the neuroprotective effect of darbepoetin is shown in rats with cerebral ischemia.[6] However, there is no data about the effects of darbepoetin-α on ethanol-induced oxidative stress. In this study, we investigated the effects of darbepoetin-α on brain tissue oxidant/antioxidant status and nitric oxide levels in experimental ethanol administration.

PATIENTS AND METHODS

This study was performed after approval from the Ethics Committee of the Animal Care Review Board of İstanbul University Cerrahpaşa Medical Faculty. Adult male Wistar albino rats, weighing 300-350 g, were obtained from Cerrahpaşa Medical Faculty Laboratory Animals' Production Center. The rats were cared for in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication no. 86-23, revised 1985), maintained in colony cages of 5 or 6 per cage, and was kept under normal conditions of temperature (23±2 °C), light (10 h light, 14 h dark), humidity (55±15% relative humidity). The rats were permitted ad libitum access to standard laboratory chow and tap water before and after experimental procedures.

Animal design

Forty-four male adult Wistar albino rats were divided randomly into four weight-matched groups: saline-treated group (S) (n=10), saline-darbepoetin-treated group (D) (n=10), experimental ethanol-administered group (E) (n=12), ethanol-administered and darbepoetin-treated group (ED) (n=12). 20% ethanol solution prepared with sterile saline was administered to rats intraperitoneally (ip) at a dosage of 2.5 g/kg (2.6 ml/kg) twice at 2-hr intervals. Darbepoetin (10 μg/kg) was administered ip. immediately after the second dose of ethanol. Application order is summarized in Table 1. Twenty-four hours after the beginning of the experiment, all the experimental animals were decapitated. The brain was quickly removed excluding the cerebellum, they were immediately immersed in liquid nitrogen and stored at -70 °C until being processed for biochemical investigation.

Biochemical procedures

Tissue homogenization. The brain tissues were weighed, washed and homogenized in ice-cold 0.9% NaCl, except GSH, which was homogenized in ice-cold 0.15 M KCl. Homogenates of 20% were obtained and sonicated twice at 30 s intervals at 4 °C. Homogenates were centrifuged at >10 000 g for 15 min at 4 °C. All biochemical parameters in homogenates were studied at the same day. Cu-Zn superoxide dysmutase (SOD), malondialdehyde (MDA), glutathione (GSH) and nitric oxide (NO) were assessed in homogenized brain tissue.

Table 1. Design of the experimental groups

	0. hour	2. hour
S (Saline-treated group) (n=10)	2.6 ml/kg saline	2.6 ml/kg saline + 10 μg/kg saline
D (Darbepoetin-treated group) (n=10)	2.6 ml/kg saline	2.6ml/kg saline + 10 μg/kg darbepoetin solution*
E (Ethanol-administered group) (n=12)	2.6 ml/kg 20% ethanol solution	2.6ml/kg 20% ethanol solution + 1 ml/kg saline
ED (Ethanol-administered, darbepoetin-treated group) (n=12)	2.6 ml/kg 20% ethanol solution	2.6 ml/kg 20% ethanol solution + 10 μg/kg darbepoetin solution*

* 1ml/kg darbepoetin solution includes 10 μg /kg darbepoetin.

Assay of MDA. Lipid peroxidation was ascertained by the formation of MDA, which was estimated by the modified thiobarbituric acid method.[9] Thiobarbituric acid-reactive substances (TBARS) concentration was calculated using 1.56_10-5M^{-1} cm^{-1} as mol/L extinction coefficient.

Assay of Cu-Zn SOD. Cu-Zn SOD activity was determined by the method of Sun et al.[10] The assay involves inhibition of nitroblue tetrazolium (NBT) (Sigma chemical Co., St. Louis, USA) and reduction with xanthine-xanthine oxidase (Sigma chemical Co., St. Louise, USA) that was used as a superoxide generator. One unit of Cu-Zn SOD is defined as the amount of protein that inhibits the rate of NBT reduction by 50%.

Assay of GSH. Tissue GSH concentration was determined according to the method of Beutler using metaphosphoric acid for protein precipitation and 50-50-dithiobis-2-nitrobenzoic acid for color development.[11] The total protein concentration of tissues was measured by the method of Lowry et al.[12]

Assay of NO. NO was measured as its stable metabolites nitrate (NO_3-) and nitrite (NO_2-). Nitrate was first reduced by nitrate reductase to nitrite and then nitrite was determined spectrophotometrically by the Griess reaction (Roche, Cat No 1 756 281).[13] Tissue NO concentrations were expressed as μmol/g wet tissue.

Data analysis

All the values were expressed as the mean ± SD. The data were analyzed by ANOVA test followed by a multiple comparison post hoc test of Tukey. Values were considered as significant when p value was less than 0.05.

RESULTS

The biochemical parameters of the experimental groups were summarized in Table 2. There were no differences between the biochemical parameters of the S and D groups. Brain tissue MDA levels of the E group were significantly higher than those of the S and D groups ($p<0.05$). The brain tissue GSH levels and Cu-Zn SOD activity of the E group were significantly lower than those of the S and D groups ($p<0.01$). Brain tissue MDA levels of the ED group were significantly lower than the E group ($p<0.05$). GSH levels of the ED group were significantly higher

Table 2. Results of biochemical paremeters in the experimental groups

	S group (n=10)	D group (n=10)	E group (n=12)	ED group (n=12)
MDA (nmol/mg protein)	0.93±0.31	0.86±0.31	2.11±0.37***,♦♦♦	1.51±0.40**,♦♦♦,•••
Cu-Zn SOD (U/mg protein)	2.01±0.30	1.95±0.31	1.21±0.36***,♦♦	1.45±0.46**,♦
GSH (μg/mg protein)	39.08±3,85	35.26±6.26	28.04±3.94***,♦♦	37.04±5.63•••
NO (μmol/mg protein)	0.40±0.01	0.42±0.01	0.55±0.01***,♦♦♦	0.45±0.01•••

• Significant differences between Sham group and other groups defined with *; ($p<0.05$)*, ($p<0.01$)**, ($p<0.001$)***

• Significant differences between D group and the other groups defined with ♦; ($p<0.05$)♦, ($p<0.01$)♦♦, ($p<0.001$)♦♦♦

• Significant differences between E group and other groups defined with •; ($p<0.05$)•, ($p<0.01$)••, ($p<0.001$)•••

than those of the E group ($p<0.001$). NO levels of the ED group were significantly lower than those of the E group ($p<0.001$).

DISCUSSION

Oxidant effects of ethanol have been reported in many studies.[1,2] It has been shown that microsomal oxidation of ethanol generates reactive oxygen species, both in vivo and in vitro, via the inducible cytochrome P450 2E1 (CYP2E1) in the the brain.[14,15] Malondialdehyde is one of the products of lipid peroxidation. Lipid peroxidation occurs when a free radical attacks the fatty-acid side chain of a phospholipid in the cellular membrane.[16] Oxidant and antioxidant status is vital for regulation of homeostasis. Reactive oxygen species (ROS), namely superoxide and hydroxyl free radicals, together with hydrogen peroxide, are believed to be directly toxic, and ROS can initiate a free-radical-mediated chain reaction that causes additional organ damage.[17] Additionally, GSH, a tripeptide and SOD are involved in the antioxidant system and are important for the protection of the tissue from oxidative damage. Oxidized form of GSH is a dimer— GSSG, which is involved in the transport of certain amino acids, and is a coenzyme for various enzymes and protects against oxygen radicals and toxic compounds. GSH removes the toxic substances from the environment and protects the tissue from harmful substances after biotransformation. High GSH activity protects the cells from oxidative damage by inhibiting lipid peroxidation.[18] SOD that catalyzes the dismutation of superoxide to hydrogen peroxide catalyses the conversion of two O_2 molecules into H_2O_2 and O_2. SOD exists in mitochondrial (Mn-SOD) and cytoplasmic forms (Cu-Zn SOD).[19] In our study, MDA and NO levels increased significantly ($p<0.001$) and the antioxidant parameters (Cu-Zn SOD, GSH) decreased significantly ($p<0.001$) after ethanol administration.

NO is a free radical gas molecule, which is produced from L-arginine by the catalytic action of enzyme, nitric oxide synthesis (eNOS, iNOS, and nNOS).[20,21] NO is a key factor in a variety of physiological processes such as neurotransmission and regulation of blood vessel wall in physiological levels.[20,22] The role of NO seems to be controversial because in some models of inflammation it has been shown that tissue dysfunction or injury could occur after inhibition of NO.[23,24] It has been suggested that increased NO is associated with oxidative stress. It has been shown that glutamate and NO promote oxidative damage by reacting with superoxide anion to form the oxidant compound peroxynitrite.[23,25-27] High production of NO has been suggested as a cause of tissue injury under certain circumstances, may be through the generation of potent free radicals. Studies are not in consensus as to whether NO is cytotoxic or cytoprotective. It may act both as a cytotoxic and/or a cytoprotective agent and the main determinants are its concentration and the environment.[28] We observed that NO levels were parallel with lipid peroxidation levels. Darbepoetin-α treatment after ethanol administration increased antioxidant response and decreased lipid peroxidation in our study.

Many different techniques and drugs are evaluated for the treatment of side effects of ethanol.[29] The data about the effects of darbepoetin-α on oxidative stress is limited. However, some protective effects of EPO against oxidative damage have been reported.[23,30] EPO treatment diminished lipid peroxidation levels and increased glutathione peroxidase activities significantly in brain tissue.[2,31,32] Antioxidant properties of darbepoetin-α in ethanol induced oxidative stress have been shown in our study. Darbepoetin-α protects brain tissue via its antioxidant activity.

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