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Effects of *Ginkgo biloba* extract on brain oxidative condition after cisplatin exposure

Abstract

Purpose: The purpose of this study was to evaluate the efficacy of *Ginkgo biloba* extract (EGb 761) on oxidative events of brain in cisplatin-administrated rats.

Methods: Rats were divided into four experimental groups: 1) control (n=6); 2) cisplatin (8 mg/kg, intraperitoneally one dose, n=6); 3) EGb 761 (100 mg/kg intraperitoneally for 15 days, n=6); and 4) cisplatin + EGb 761 (n=6). After drug administration, rats were sacrificed and brain tissues were removed. Nitric oxide (NO), malondialdehyde (MDA) and glutathione (GSH) levels were evaluated in brain tissues.

Results: Single dose cisplatin administration significantly increased NO and GSH levels, but decreased MDA levels in brain tissue samples. EGb 761 treatment reversed the effects of cisplatin on NO and GSH levels, but did not affect the decreased MDA levels.

Conclusion: Results of the study indicate that oxidative stress can be an important pathogenetic mechanism of cisplatin-induced neurotoxicity. EGb 761, an standardized extract of *G. biloba* leaves that has antioxidant properties, may improve the oxidative stress-related neurological side effects of cisplatin.

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Cisplatin (CDDP) was the first platinum derivate that was used as an anticancer "antineoplastic" or "cytotoxic" drug and its beneficial effects have been shown in various cancerous diseases including bladder carcinoma, small cell bronchial carcinoma, and tumours in testes, ovary, endometric and prostatic cells [1]. Its clinical use is limited by its severe side effects, which include nephrotoxicity, ototoxicity, bone marrow toxicity, gastrointestinal toxicity and peripheral neurotoxicity [2,3]. Peripheral neurotoxicity is the most significant dose-limiting problem associated with CDDP use [4]. CDDP induces peripheral neurotoxicity that mainly affects the large myelinated nerve fibres of the dorsal root ganglia [5,6,7]. Neuropathologic examinations in animal models of CDDP have revealed pathologic changes in the dorsal-root ganglia [8]. Similarly, pharmacological examinations of neural tissue of patients treated with cisplatin have shown platinum accumulation in the dorsal-root ganglia that are not protected by the blood-brain-barrier [9].

Reactive oxygen species (ROS), including superoxide radical, hydrogen peroxide, hydroxyl radical and singlet oxygen, are generated during normal cellular metabolism [10,11]. Physiological levels of ROS can be scavenged by enzymatic (e.g., superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR)) and non-enzymatic (e.g., vitamin C, vitamin E and glutathione) antioxidant defence systems. Excessive ROS levels indicate decreased antioxidant defence ability or increased oxidative stress. Excess ROS reacts with nitric oxide (NO) to generate reactive nitrogen species (RNS) such as peroxynitrite [12]. ROS affect cell functions by directly acting on cell components, including lipids, proteins and DNA, and damaging their structure [13]. ROS are particularly active in the brain and neuronal tissue, and are involved in numerous cellular functions, including cell death and survival. A high metabolic rate and an abundant supply of the transition metals make the brain an ideal target for a free radical attack. In addition, the brain has a high susceptibility to oxidative stress due to the high lipid content and relatively lower regenerative capacity in comparison with other tissues. Oxidative stress is involved in the pathogenesis of a number of neurologic conditions and neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and epilepsy [14-16]. An imbalanced overproduction of ROS can induce neuronal damage, leading to neuronal death by necrosis or apoptosis. Recent evidence indicates that cisplatin-induced side effects are, at least in part, due to the result of formation of ROS [17, 18].

Antioxidants are considered to be promising approaches to neuroprotection. Previous studies were demonstrated effects of antioxidants on neurodegenerative disorders *in vitro* and in animal models [19-23]. A number of studies have focused on the role of antioxidants in cisplatin toxicity. In animals, administration of antioxidants, such as vitamin E, vitamin C and selenium, seems to protect against cisplatin-induced renal and ototoxicity [24]. Also, the data obtained from human studies point that cisplatin treatment induces a significant decrease in plasma antioxidant levels because of oxidative stress [17].

The leaves of *Ginkgo biloba* tree have been used in traditional Chinese medicine for several hundred years. EGb 761, a standardized extract of *G. biloba* leaves, is reported to alleviate symptoms in (or has been shown to have neuroprotective effects in) various central nervous system (CNS) disorders, such as dementia, cerebral insufficiency, ischemia, traumatic brain injuries, retinal degeneration, vestibular dysfunction and anxiety. It has also been reported to enhance mental and cognitive functions in experimental animals and human subjects [25]. One of the suggested mechanisms for its efficacious pharmacologic effects is its potent antioxidant action [26]. Studies have shown the protective effects of EGb 761 against oxidative stress, and these are closely related to its ability to scavenge free radicals, such as superoxide anion, hydroxyl, peroxy radicals and nitric oxide [27-30]. EGb 761 has also been shown to prevent lipid peroxidation and enhances antioxidant enzyme activities [31]. The aim of the present study was to examine the effects of EGb 761 administration on the oxidative condition of rat brain tissue following cisplatin exposure.

Materials and Methods

Animals and experimental groups

Healthy adult female Wistar rats (n=24) were used for the study. All rats had free access to commercial food and water and were maintained in an environment with controlled temperature and 12-hour light/dark cycles. All experiments were conducted with governmental approval according to local guidelines for the care and use of laboratory animals and the guidelines of the European Community Council for experimental animal care.

The rats were divided into four experimental groups: 1) control (n=6); 2) cisplatin (8 mg/kg, intraperitoneally one dose, n=6); 3) EGb 761 (100 mg/kg intraperitoneally for 15 days, n=6); and 4) cisplatin + EGb 761 (cisplatin 8 mg/kg, intraperitoneally one dose; EGb 761 100 mg/kg

intraperitoneally for 15 days) ($n=6$). Under the urethane anaesthesia (1.2 g/kg, intraperitoneally) rats were sacrificed and brain tissues were removed, immediately frozen in liquid nitrogen and kept at -30°C until assayed.

Determination of MDA Levels

Malondialdehyde (MDA) levels of brain tissue were determined according to the spectrophotometric method of Casini *et al.* (1986). Tissue samples were homogenized in nine volumes of cold 10% TCA solution and the homogenates were centrifuged for 15 min at 3000 g at 4°C . The supernatants were transferred to glass test tubes containing 0.375% (w/v) thiobarbituric acid and 0.02% (w/v) butylated hydroxytoluene to prevent further lipid peroxidation during the subsequent steps. The samples were then heated for 15 min at 100°C in a boiling water bath, cooled and centrifuged to remove the precipitant. The absorbance of each sample was determined at 532 nm.

Determination of Glutathione Levels

Glutathione (GSH) levels were determined by the modified Elman method (Aykaç *et al.* 1985). Tissue samples were homogenized in ice-cold trichloroacetic acid (1 g tissue plus 10 ml 10% trichloroacetic acid) in tissue homogenizer. After centrifugation at 3000 g for 10 minutes, 0.5 ml of supernatant was added to 2 ml of 0.3 M $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ solution. Then 0.2 ml of dithionitrobenzoic acid solution (0.4 mg/ml 1% sodium citrate) was added and the absorbance at 412 nm was measured immediately after mixing. The GSH levels were calculated using an extinction coefficient of 13.600 mol/cm.

Determination of NO Levels

NO values are given by the sum of nitrite and nitrate, which are the stable end products of nitric oxide. NO levels in tissue samples were determined by the Griess reaction (Green *et al.* 1982). The tissue samples were homogenized in five volumes of phosphate buffer (pH 7.5) and centrifuged at 2000 g for 5 min and (0.5 ml) 0.25 ml of 0.3 M NaOH were added to the supernatants. After incubation for 5 min at room temperature, 0.25 ml of 10% (w/v) ZnSO_4 was added for deproteinization. This mixture was then centrifuged at 14000 g for 5 min, and then the supernatants were used for the Griess assay. The nitrate levels in tissue homogenates were determined spectrophotometrically, based on the reduction of nitrate to nitrite by VaCl_3 (Miranda *et al.* 2001). The nitrite levels were measured by the Griess reaction. Sodium nitrite and nitrate solutions (1, 10, 50, 100 μM) were used as standards.

Statistical analyses

Statistical procedures were performed using SPSS statistical software package (version 12.0; SPSS Inc., Chicago, IL, USA). All analyses were carried out by one-way analysis of variance (ANOVA), followed by Bonferroni *post hoc* test to correct for multiple comparisons of treatments. Data are expressed as the mean $\pm$ the standard error of the mean (SEM). The significance level was $p < 0.05$.

Results

The control level of NO in brain was found as 149.5 ± 7.9 $\mu\text{mol/g}$ tissue. Figure 1 shows that NO level were increased significantly in cisplatin group ($p < 0.001$) (Table 1); however, NO levels of EGb 761 and Cisplatin+EGb 761 groups decreased significantly compared with control ($p < 0.001$) and cisplatin ($p < 0.001$) groups. In the control group, the brain level of GSH was 3.1 ± 0.3 $\mu\text{mol/g}$ tissue. Figure 2 shows that GSH levels increased in all groups, compared with controls. The changes of GSH levels in cisplatin, cisplatin+EGb 761 ($p < 0.01$) and EGb 761 ($p < 0.05$) groups were statistically significant. Assessment of brain MDA levels showed that the control level was 103 ± 7.1 nmol/g tissue. Figure 3 shows that, brain MDA levels decreased in both cisplatin and cisplatin+EGb 761 groups compared with the control group ($p < 0.001$). There were no significant changes in EGB 761 group compared with the control ($p > 0.05$) group.

Discussion

The present study evaluated the efficacy of EGb 761 on oxidative events in brains of cisplatin-administrated rats. Cisplatin is classified as an alkylating agent. Because alkylating

FIGURE 1. NO levels of brain according to groups. ^a $p < 0.001$, ^b $p < 0.01$, ^c $p < 0.05$, as compared with control group by Bonferroni *post hoc* test

FIGURE 2. GSH levels of brain according to groups. ^a $p < 0.001$, ^b $p < 0.01$; ^c $p < 0.05$, as compared with control group by Bonferroni *post hoc* test

agents are most active in the resting phase of the cell, neuronal tissue is a target of their side effects. Cisplatin is reported to cross the blood-brain barrier only in limited amounts, as the brain-blood barrier limits protein-bound cisplatin penetration. In one study, cisplatin administration resulted in a peak CSF concentration as high as 40% of nonprotein bound cisplatin [32]. Despite the overall poor penetration of cisplatin across the brain-blood barrier, a number of CNS complications (including encephalopathy and epilepsy) may occur [33].

Our findings showed that cisplatin treatment increase NO levels of brain. The probable mechanism is that cisplatin elevated iNOS activity and induced the generation of large amounts of NO. Some investigators have shown that cisplatin increases inducible NO synthase (iNOS), which results in an increase in NO release [34-36]. Also, EGb 761 decreased NO levels when applied to the rats subjected to cisplatin. Kobuchi *et al.* [37] have reported that EGb 761 inhibits the enzymatic activity of iNOS. Consistent with previous studies, data from our study show a marked elevation in NO levels in brain tissue of cisplatin-administrated rats and this elevation in NO levels was significantly decreased by EGb 761.

The increase of ROS, which attack the cell membrane lipids, occurs due to increased tissue lipid peroxidation as manifested by increased MDA levels. Administration of cisplatin causes an increase in lipid peroxidation and a decrease in enzymatic and non-enzymatic antioxidants that prevent or protect against lipid peroxidation in tissues. GSH is one of the non-enzymatic antioxidants that maintaining cell integrity because of its reducing specifications and accession in the cell metabolism. Several researchers have demonstrated increased lipid peroxidation and decreased antioxidant levels induced by cisplatin treatment in various tissues [38, 39, 40, 36]. In

FIGURE 3. MDA levels of brain according to groups. ^a $p < 0.001$, ^b $p < 0.01$; ^c $p < 0.05$, as compared with control group by Bonferroni *post hoc* test

contrast to these findings, we found higher GSH levels and lower MDA levels in the cisplatin group than in the control animals. A possible explanation for the reduction of MDA concentration may be enhanced formation of antioxidants in the brain tissue, which, due to the activity of ROS, would allow a consequent decrease in the production of MDA. The underlying mechanism of cisplatin-induced elevation in GSH level remains unclear. On the other hand, Yilmaz and coworkers [35] showed that cisplatin increased NO levels, with no change in MDA levels, in liver tissue in rats.

Some investigators have reported that EGb 761 protects several types of neurons in central nervous system, including cerebral cortex [41], cerebellum [42] and hippocampus [43] in conditions such as oxidative stress and ischemia. In the CNS, different mechanisms of action have been suggested for EGb 761, including antioxidant, anti-ischemic, anti-apoptotic and neurotransmitter modulatory effects [25]. Antioxidant effects of EGb 761 are related to its ability to acts as a free radical scavenger by preventing lipid peroxidation, enhancing GSH levels and GSH reductase activity [44]. The current study indicates that the administration of EGb 761 elevated GSH levels and decreased MDA levels significantly in cisplatin+EGb 761 group compared with controls. Similar to our finding, Xu *et al.* [45] showed that EGb 761 attenuated cisplatin-induced changes in serum superoxide dismutase (SOD) activity and MDA levels in guinea pigs. Also Güleç and colleagues [38] reported that EGb 761 administration increased GSH-Px activity and decreased NO levels in serum samples of cisplatin administrated rats.

In conclusion, this study suggests that *Ginkgo biloba* extract-EGb 761 protects against cisplatin-induced oxidative damage in the brain; however, further studies are required to

reveal the mechanisms of these protective effects using different doses of EGb 761 and different experimental models.

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