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In vivo protective effect of Uridine, a pyrimidine nucleoside, on genotoxicity induced by Levodopa/Carbidopa in mice

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ABSTRACT

Parkinson's disease (PD) is a common neurodegenerative disorder that affects millions of people all over the world. Motor symptoms of PD are most commonly controlled by L-3,4-dihydroxyphenylalanine (Levodopa, L-DOPA), a precursor of dopamine, plus a peripherally-acting aromatic-L-amino-acid decarboxylase (dopa decarboxylase) inhibitor, such as carbidopa. However, chronic treatment with a combination of Levodopa plus carbidopa has been demonstrated to cause a major complication, namely abnormal involuntary movements. On the other hand, the effect of this treatment on bone marrow cells is unknown. Therefore, in this study, we aimed to investigate possible genotoxic effects of Levodopa and Carbidopa using male Balb/C mice. Our results showed that Levodopa alone or in combination with carbidopa caused genotoxicity in *in vivo* micronucleus test (mouse bone marrow) and Comet assay (blood cells). Furthermore, we showed that simultaneous administration of uridine, a pyrimidine nucleoside, reversed the genotoxic effect of Levodopa and Carbidopa in both assays. Our data show for the first time that Levodopa plus carbidopa combination causes genotoxicity which is reversed by uridine treatment. These findings might enhance our understanding for the complications of a common Parkinson's treatment and confer benefit in terms of reducing a possible genotoxic effect of this treatment.

1. Introduction

Parkinson's disease (PD) is one of the most common neurodegenerative disorders which affect at least four million people worldwide. It is characterized by the progressive degeneration of dopaminergic nigrostriatal neurons, and reductions in striatal dopamine (DA) levels (Cansev et al., 2008; Cornetta et al., 2009). Although oxidative stress seems to play a major role in PD pathogenesis (Cornetta et al., 2009; Jenner, 2003; Melamed et al., 2000; Müller et al., 2004; Snyder and Friedman, 1998), the pathogenic mechanisms underlying the disease are still indefinite.

L-3, 4-dihydroxyphenylalanine (Levodopa, L-DOPA), the natural metabolic precursor of dopamine, is the gold standard drug for symptomatic treatment of Parkinson's disease (Mena et al., 1992). Despite the conspicuous benefits of Levodopa treatment, a major problem with the treatment is its potential neurotoxicity (Koh et al., 2009). In PD, Levodopa is almost always administered in combination with a peripherally-acting aromatic-L-amino-acid decarboxylase (dopa decarboxylase) inhibitor, such as carbidopa, which maximizes the amount of levodopa available to enter the brain (Basma et al., 1995; Bugamelli et al., 2011).

In recent years, several studies have demonstrated that Levodopa is toxic to various cells *in vitro* (Mena et al., 1992; Mytilineou et al., 1993; Pardo et al., 1995). In accordance, Snyder and Friedman (1998) reported that antiproliferative and clastogenic activity of Levodopa is enhanced by concomitant exposure to manganese chloride or copper sulfate in Chinese Hamster V79 cells. Consequently, it has been suggested that the toxic nature of Levodopa might affect human health in a negative manner. Several recent studies have provided evidence that long-term administration of Levodopa to patients with Parkinson's disease increased the degree of neuronal damage and thus accelerated the progression of the disease (Müller et al., 2004; Weiner, 2006). The well-defined motor complication, Levodopa-induced dyskinesia (LID), is a form of dyskinesia which involves hyperkinetic movements including chorea, dystonia, and athetosis, and is associated with Levodopa used to treat PD (Gerlach et al., 2011).

Enhanced oxidative stress has been suggested as one of the mechanisms for potential Levodopa toxicity (Graham et al., 1978; Snyder and Friedman, 1998). Enzymatic degradation or autooxidation of levodopa has been shown to generate a variety of toxic free radical species including superoxide radical, hydrogen peroxide (H_2O_2), hydroxyl radical, quinones and semiquinones (Fahn and Cohen, 1992;

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Graham et al., 1978; Olanow et al., 2004; Ziv et al., 1997). These metabolites bear the potential to damage critical biomolecules such as DNA, proteins, and lipids, and to cause cell death (Jenner, 2003). Increased generation of cell toxins (Maharaj et al., 2005) such as salsolinol (Jung et al., 2001) or 6-hydroxydopamine (6-OHDA) (Blunt et al., 1993; Koh et al., 2009; Maharaj et al., 2005) as well as induction of cell membrane lipid peroxidation and rupture (Olanow, 1993; Ziv et al., 1997), inhibition of mitochondrial electron-transport chains (Pardo et al., 1995), and induction of apoptotic cell death with cell shrinkage, membrane blebbing and DNA fragmentation (Walkinshaw and Waters, 1995; Ziv et al., 1997) have also been suggested to be involved in Levodopa toxicity.

Uridine is a circulating pyrimidine nucleoside and it is found in the tissues (Cansev, 2006; Lecca and Ceruti, 2008). Exogenous administration of uridine has been shown to provide protection in several disease models such as ischemic heart disease (Lin et al., 1997) and Huntington's disease (Saydoff et al., 2006). In addition, we have recently shown that uridine might protect brain cells from hypoxia–ischemia induced damage by reducing apoptosis in a newborn rat model of hypoxic–ischemic encephalopathy (HIE) (Cansev et al., 2013).

In the present study, we have evaluated the genotoxic effects of Levodopa, the potential genotoxic/antigenotoxic effect of uridine and potential protective effect of uridine with Levodopa-associated genotoxicity using *in vivo* alkaline Comet and micronuclei assays. For this purpose, the bone marrow cells and peripheral blood lymphocytes were used in micronuclei and comet tests, respectively.

To the best of our knowledge, this is the first report on potential anti-genotoxic and protective effects of uridine on Levodopa-associated DNA damage *in vivo*.

2. Materials and methods

2.1. Animals

Seventy-six male (8- to 10-week-old) Balb/c mice weighing 25–30 g were used for this study. The mice were given free access to fresh standard pellet and tap water. Four mice were housed per cage. All mice were kept under constant environmental conditions with a 12–12 h light–dark cycle. The study was conducted in accordance with ethical procedures and policies approved by the Animal Care and Use Committee of Uludag University, Bursa, Turkey (No: 2010-02/06).

2.2. Test chemicals

Levodopa (L L-3,4-Dihydroxyphenylalanine methyl ester hydrochloride, Cas no: 1421-65-4), Carbidopa (S-(−)-Carbidopa, Cas no: 28860-95-9) and uridine (Cas no: 58–96-8) were purchased from Sigma-Aldrich Chemical Company and the purities of these compounds are 98 and ≥99%.

Other chemicals; Phosphate buffered saline (PBS), NaCl, Na_2EDTA, Tris, Triton X100, DMSO, HCl, NaOH, Normal Melting Agarose, Low Melting Agarose, May–Grünwald's eosine-methylene blue solution, Giemsa stain solution, Fetal Calf Serum (FCS) and Ethyl Methane Sulfonate (EMS) were also purchased from Sigma-Aldrich Chemical Company.

2.3. Doses and treatments

The intraperitoneal (i.p.) route of application was used in all experiments. We first tested Levodopa doses of 10, 50, 100, 250, 500 and 1000 mg/kg. The higher doses (250 and 500 mg/kg) caused a dramatic decrease on proliferation index of bone marrow cells using micronucleus assay. Mice were followed up for a period of 24 hours for death assessment and 1000 mg/kg dose caused death within this period. Comet test cell viability assessment was performed immediately after sacrificing the mice when the majority of cells were viable. Therefore, the 10, 50, and 100 mg/kg doses of Levodopa were chosen for this study.

Levodopa was administered in combination with carbidopa, an inhibitor of dopa decarboxylase. Since the combination drugs used for Parkinson's treatment and marketed in Turkey include one unit carbidopa to ten units Levodopa, carbidopa was administered at 1, 5 and 10 mg/kg doses.

We also tested 100, 200, 400 and 1000 mg/kg doses of uridine, and, in the light of our preliminary observations, a single dose of uridine, 100 mg/kg, was chosen for the study.

Each drug was administered intraperitoneally (i.p.) in 0.1 mL volume. Distilled water and acidified water (0.003 N HCl) were administered i.p. in the same volume as negative and solvent control, respectively. A single dose (300 mg/kg) of EMS was used as a positive control.

Mice were randomized to nine treatment groups as follows: 1) Levodopa alone in three different doses (10, 50 and 100 mg/kg; n = 12); 2) Carbidopa alone in three different doses (1, 5 and 10 mg/kg; n = 12); 3) Levodopa plus Carbidopa (10/1, 50/5, 100/10 mg/kg; total n = 12); 4) Uridine alone (100 mg/kg; n = 4); 5) Levodopa in three doses (10, 50 and 100 mg/kg) plus uridine (100 mg/kg) (total n = 12); 6) Levodopa/Carbidopa in three different dose combinations (10/1, 50/5 and 100/10 mg/kg) plus uridine (100 mg/kg) (total n = 12); 7) Negative control (distilled water) group (n = 4); 8) Solvent (acidified water) control group (n = 4); and 9) Positive control (EMS) group (n = 4). The analyses were performed 24 h after the drug treatments.

2.4. Micronucleus assay

Femurs of mice were dissected and the marrow was aspirated into a 2 mL fetal bovine serum. The cells were then mixed thoroughly to obtain a fine suspension and the latter was centrifuged at 800–1000 rpm for 5 min. The pellet was then resuspended in fresh bovine albumin and smears were prepared on clean glass slides. The smear slides were stained with May–Grünwald plus Giemsa at pH 6.8 (Mac-Gregor et al., 1987). For the determination of the frequency of micronucleated polychromatic erythrocytes (MNPCEs), 1000 polychromatic erythrocytes (PCEs) per animal were analyzed by light microscopy under oil immersion. Cytotoxicity was assessed by scoring the relative proportion of polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE). This ratio was determined by counting a total of 2000 erythrocytes for each animal.

The percent reduction in the frequency of MNPCEs was calculated according to Waters et al. (1990), using the following formula:

$$\text{Reduction}\,(\%) = \frac{\text{no. of cells with MN in A} - \text{no. of cells with MN in B}}{\text{no. of cells with MN in A} - \text{no. of cells with MN in C}} \times 100$$

where A corresponds to the group with Levodopa or Levodopa/Carbidopa treatment (positive control), B to the group treated with Levodopa or Levodopa/Carbidopa plus uridine and C corresponds to the group treated with distilled water or solvent (negative control).

2.5. Single cell electrophoresis (Comet) assay

The alkaline Comet assay was performed as described by Cornetta et al. (2009) with minor modifications. One hundred microliters of whole blood/heparin mixture were mixed in 250 μL of 0.7% low melting point agarose in PBS (Ca and Mg free) at 37 °C, and immediately pipetted onto a frosted glass microscope slide precoated with a layer of 0.8% normal melting point agarose, similarly prepared in phosphate-buffered saline (PBS). Two slides were prepared for each experimental point. The slides were put in to a lysis solution (2.5 M NaCl, 10 mM Tris–HCl, 100 mM Na2EDTA, NaOH to pH = 10, 1% Triton, 10% dimethyl sulfoxide [DMSO]) for overnight incubation. After lysis, the slides were placed onto a horizontal electrophoresis unit containing fresh running buffer (1 mM Na2 EDTA, 300 mM NaOH, pH = 13) for 20 min to allow DNA unwinding. Electrophoresis was conducted for 25 min (25 V, 300 mA) at +4 °C. Subsequently, the slides were gently neutralized in a buffer solution for 5 min and then stained with ethidium bromide. The stained slides were visualized under a microscope with a fluorescence attachment. The images were analyzed by using specialized software for COMET analysis (Argenit, Istanbul, Turkey). If breaks are present on nuclear DNA molecule, they are drawn out to form a "tail." To quantify the induced DNA and cell damage, the tail length, the tail DNA, which is a measure of the percentage of migrated DNA in the tail (Collins, 2004), a genetic damage index (GDI) and the percentage of damaged cells were analyzed. Genetic Damage Index (GDI) was calculated according to the following formula used by COMET analysis program (Anderson et al., 1994),

$$\text{GDI} = (1 * \Sigma\text{Type1}) + (2 * \Sigma\text{Type2}) + (3 * \Sigma\text{Type3}) + (4 * \Sigma\text{Type4}) / (\Sigma\text{Type0} + \Sigma\text{Type1} + \Sigma\text{Type2} + \Sigma\text{Type3} + \Sigma\text{Type4})$$

Percentage of Damaged Cell (PDC) was calculated according to the following formula (Anderson et al., 1994):

$$\%\ \text{Damaged Cell} = \Sigma\text{Type2} + \Sigma\text{Type3} + \Sigma\text{Type4}$$

where ΣType0 is the total not damaged cells, ΣType1 is the total very low damaged cells, ΣType2 is the total low damaged cells, ΣType3 is the total high damaged cells, ΣType4 is the total very high damaged cells (Fig. 1).

3. Results

The results of the micronuclei and comet assays are given in Tables 1 and 2.

The mouse bone marrow micronuclei assay showed that the mean micronucleus frequency was increased significantly with all three doses (10, 50, 100 mg/kg) of Levodopa compared to the