The Role of Ionic Homeostasis in Cisplatin-Induced Neurotoxicity: A Preliminary Study

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ABSTRACT

Objective: The aim of the present study was to investigate the role of ionic homeostasis in cisplatin (cis-diamminedichloroplatinum (II), CDDP)-induced neurotoxicity. CDDP is a severely neurotoxic antineoplastic agent that causes neuronal excitotoxicity. According to some studies, calcium influx increases, whereas potassium efflux decreases neuronal death. Nimodipine and glibenclamide were used to analyze the role of ionic flows in CDDP-induced neurotoxicity in rat primary cerebellar granule cell (CGC) culture.

Materials and Methods: CGC culture was prepared from the cerebella of Sprague Dawley 5-day-old pups. The submaximal concentration of CDDP was determined and then given with 1, 10, or 50 μM of drugs into culture. Neurotoxicity was investigated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) assay. One-way analysis of variance, Kruskal–Wallis H test, and Tukey test were applied for statistical analysis.

Results: CDDP induced neurotoxicity in a concentration-dependent manner. Neither nimodipine nor glibenclamide was able to protect CGCs against CDDP neurotoxicity.

Conclusion: By blocking L-type voltage-gated calcium channels, nimodipine did not prevent CDDP neurotoxicity in CGCs. Ca^{2+} influx via these channels seemed to be insufficient to cause a change in CDDP-induced neurotoxicity. Similarly, glibenclamide failed to prevent CDDP neurotoxicity. Further studies are needed to elucidate the mechanisms of these preliminary results.

Keywords: Cerebellar granule cells, glibenclamide, KATP channels, nimodipine

Introduction

Cisplatin (cis-diamminedichloroplatinum (II), CDDP) is a platinum-based antineoplastic agent widely used against a variety of cancers [1]. However, its clinical benefits are limited owing to its adverse effects, such as ototoxicity, nephrotoxicity, cardiotoxicity, hepatotoxicity, and central and peripheral neurotoxicity [2]. Neurotoxicity is an important dose-limiting adverse effect that may cause not only dose reduction or drug cessation during therapy but also reduction of the quality of life of patients. The neurotoxicity induced by CDDP is found to have active neuronal cell death features and also has glutamate-dependent excitotoxic features [3]. CDDP affects calcium homeostasis and causes increased intracellular calcium concentrations [4]. However the knowledge about the neurotoxicity of cerebellar granule cells (CGCs) induced by CDDP is limited.

The ion movements are crucial for neurotoxic or neuroprotective mechanisms in antineoplastic agent therapy. Particularly, calcium and potassium ions may have critical roles in neuronal cell death [5]. Calcium ions are fundamental for performing normal cellular functions and for cellular survival. However, calcium concentration may reach critical levels in pathological conditions, such as ischemia or brain damage, leading to cellular damage or cell death [6, 7]. Increased intracellular calcium levels are caused by release from internal stores and calcium influx via channels in the membrane. The dysregulation of calcium homeostasis and calcium signaling is known to contribute to the neurotoxic side effects of CDDP. Particularly, voltage-gated calcium channels are believed to play a role in CDDP-induced neurotoxicity [4]. Thus, the aim to buffer excess intracellular calcium may be achieved by using calcium antagonists that block voltage-gated calcium channels [8].

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K_{ATP} channels belong to Kir channels characterized by inward rectification by allowing more current to flow inward than outward. These channels are important ion channels that regulate many cellular functions, neuronal signaling, and membrane excitability according to both electrical activity and metabolic status of the cell. In many cell types, it was shown that they may have a role in neuroprotective mechanisms [9]. However, there is a controversy in the results of studies about the contribution of these channels to neuroprotective strategies. In previous studies, the blockade of K_{ATP} channels protects the dopamine neurons from degeneration; however, the activation of K_{ATP} channels can also protect mesencephalic neurons from MPP^+ cytotoxicity [10, 11]. Moreover, K_{ATP} channel activation was found to protect CGCs from oxidative stress [11].

In previous CGC neurotoxicity studies, neuronal death was shown to arise from several reasons, such as excess glutamate release, changes in Ca^{2+} homeostasis, deficiency of K^+, reactive oxygen species production, and caspase activation [8, 12]. Any change in the concentrations of these molecules may cause cell death. Although molecular mechanisms of pathological cell loss have been extensively investigated in many studies, the contribution of the disruption of ionic homeostasis to cell death is still unknown. However, excitotoxicity was found to be increased by calcium influx and decreased by potassium efflux in different cell types [13].

The evaluation of neurotoxicity can be achieved by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) assay, which is widely used in cytotoxicity studies. The MTT assay is a colorimetric viability test based on the enzymatic reduction of the MTT (thiazolyl blue tetrazolium bromide) molecule to formazan crystals in the presence of viable cells. This enzymatic reduction concludes with a change in color that can be easily detected by an ELISA reader system in terms of absorbance values [14].

CDDP was found to activate excitotoxic mechanisms and also trigger active neuronal death [3]. It is widely known that calcium influx increases, whereas potassium efflux decreases excitotoxicity [13]. The aim of this preliminary study was to evaluate the potential roles of the channels that regulate calcium and potassium ions in the neurotoxicity of CDDP. To determine the effect of calcium ions, the cells were treated with nimodipine (L-type calcium channel blocker). The cells were also treated with glibenclamide (K_{ATP} channel blocker) to evaluate the potential therapeutic effect of K_{ATP} channels in CDDP neurotoxicity.

Materials and Methods

Primary cultures of CGCs were prepared from 5-day-old newborn Sprague–Dawley rats with modifications in the method described by Xu and Wojcik [15]. Approval was obtained from the local ethics committee for animal experimentations. In addition, informed consent was obtained from the participants of the study, and the institution's ethics committee approved the study (protocol number: 273/2012). The animals were procured from the Medical and Surgical Research Center in Eskişehir Osmangazi University. Briefly, newborn rats were decapitated, and the cerebellum was removed. To prevent contamination, the cerebellum was washed twice with calcium-free Hank's Balanced Salt Solution (HBSS) (HyClone Thermo Scientific, Germany). Then, it was suspended in 5 ml calcium-free HBSS containing 2 ml of trypsin–EDTA (0.25% trypsin–0.02% EDTA; Sigma Aldrich, USA) at 37°C for 40 min. Trypsin digestion was attenuated by adding 5 ml of fresh Dulbecco's Modified Eagle Medium (DMEM) (Sigma Aldrich, Germany). DMEM solution comprised 10% fetal calf serum (FCS) (HyClone Thermo Scientific, Germany), 50 µg/mL penicillin, and 0.2 mM L-glutamine (Sigma Aldrich, Germany). After centrifugation at 1000 rpm for 5 min, the resulting pellets were suspended. After enzymatic disintegration, the cell suspension was pipetted up and down using Pasteur pipets with soft-headed tips that provide mechanic disintegration called trituration.

The bases of 25 cm^2 polypropylene tissue culture flasks were covered with poly-D-lysine (Sigma Aldrich, Germany). Twenty-four hours before the experiment, 0.1 mg/mL poly-D-lysine (30,000–70,000 MW) was dissolved in phosphate-buffered saline, and the bases of 96-well plates were also covered with the same solution. After keeping at room temperature (25°C) for 5 min, excess poly-D-lysine was vacuumed and left to dry at 4°C overnight. Then, the cell suspension was transferred into covered flasks to allow adherence to the surface, and culture flasks were incubated at 37°C and humidified at 95% air and 5% CO_2. After 30 min, the media was changed to eliminate non-adherent cells, and fresh DMEM containing 10% FCS was added into the flasks. Culture media was changed twice a week, and neurons were used for neurotoxicity experiments following an 8-day in vitro incubation.

The dye exclusion method was applied to stain the cells with 0.4% trypan blue for counting live cells. An inverted light microscope was used to examine stained and non-stained cells immediately. Approximately 10,000 cells per well were plated in 96-well culture plates in drug-free DMEM medium overnight. CDDP (100, 200, and 500 µM) (Sigma Aldrich, USA) was applied to wells at gradually increasing concentrations. After applying the drug into cell suspensions, the plates were incubated overnight at 37°C and humidified at 95% air and 5% CO_2. The toxic effects of CDDP were evaluated using the drug alone or with nimodipine (Sigma Aldrich, USA) or glibenclamide (Sigma Aldrich, USA) at concentrations of 1, 10, and 50 µM.

The MTT assay was used for cytotoxicity determination. MTT was dissolved in HBSS at a final concentration of 1 mg/mL. Briefly, at the end of the incubation period, the cells inside the 96-well culture plates were incubated with MTT solution at 37°C for 4 h. After vacuuming the incubation solution, the cells were lysed with 150 µL dimethyl sulfoxide and subjected to the measurement of optical density at 540 nm as reference on an ELISA system (Thermo Lab Systems Multiscan EX, USA) [16, 17].

Results were evaluated using the ELISA reader system (Thermo Lab Systems, Germany). Drug applications to 96-well plates were handled at different times so each plate was evaluated according to its control data. Data were analyzed using SPSS 15.0 statistical package program (IBM SPSS Inc., Chicago, IL, USA). The percentage of cell death scores was calculated, and the results were statistically analyzed using Kruskal–Wallis H and Tukey tests. A p-value<0.05 was considered significant.

Results

The percentage of cell death according to each group was calculated using the following formula: [relative cell death = 1 − (n / nK ort)]. As expected, CDDP induced neuronal cell death concentration dependently (Figure 1). The minimum inhibitor concentration of CDDP was determined as 200 µM ($p<0.01$) (Table 1, Figure 1). Nimodipine and glibenclamide combinations were performed together with 200 µM of CDDP.

The difference between the CDDP group and the CDDP–nimodipine combination groups was not significant. In addition, nimodipine did not induce any toxicity at all concentrations (1, 10, and 50 µM). Thus, nimodipine neither increased nor decreased the neurotoxicity of CDDP (Table 2, Figure 2). CDDP neurotoxicity did not significantly change when it was applied together with glibenclamide. However, 10 µM

glibenclamide increased CDDP neurotoxicity ($p<0.001$). In addition, glibenclamide alone was not toxic to the CGCs at all concentrations applied (Table 2, Figure 3).

Discussion

The present study investigated the effects of L-type Ca^{2+} channel blocker nimodipine and K_{ATP} channel blocker glibenclamide in CDDP-induced neurotoxicity using primary CGCs. Neither nimodipine nor glibenclamide significantly changed CDDP neurotoxicity.

An increase in intracellular calcium concentration is a common mechanism for the neurotoxic side effects of CDDP. Calcium channels are mainly of L-type (60%–70%) in 1-week old CGC culture [18]. Thus, calcium influx via these channels may be believed to be critical for neuronal cell death. However, our results were quite the opposite. The blockade of L-type calcium channels via nimodipine could not protect the cells. Neuronal L-type calcium channels have different gating properties identified in a variety of neurons, including CGCs [19]. This difference in gating properties of L-type calcium channels may be due to nimodipine ineffectiveness. Moreover, nimodipine-sensitive L-type current contributed to 15% of the total current, and the prevention of calcium influx via nimodipine-sensitive calcium channels may be inadequate to prevent CDDP neurotoxicity [20].

The other calcium influx routes may be responsible for CDDP neurotoxicity rather than the L-type calcium channels. In the past, an increase in intracellular calcium was known to be responsible for neurotoxicity, whatever way calcium goes into the cell; however, recent studies focused on the way of calcium influx and the involved second messenger systems [21]. Similarly, in hippocampal neurons, calcium influx via L-type calcium channels was found to be harmless, but calcium influx via N-methyl-D-aspartate (NMDA) receptors was toxic for the cells [22]. Thus, the blockade of L-type calcium channels was suggested to be unable to prevent toxic calcium influx in our study. The increase in toxicity may be relevant to other calcium routes (e.g., NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, kainate receptors, and

Table 1. Concentration-dependent cytotoxicity induced by cisplatin (Cis100, Cis200, and Cis500: cisplatin 100 µM, 200 µM, and 500 µM, ***p<0.001)

		n	Mean±SE	Median (25%–75%)	p*	Pairwise comparisons
1	Control	33	0.265±0.028	0.240 (0.115–0.360)		
2	Cis100	23	0.351±0.036	0.388 (0.193–0.450)	<0.001	1–3***, 1–4***
3	Cis200	72	0.591±0.017	0.619 (0.497–0.701)		
4	Cis500	24	0.759±0.006	0.760 (0.749–0.782)		

*Kruskal–Wallis one-way analysis of variance on ranks (median 25%–75%)

Table 2. Relative cell death values for the cisplatin/nimodipine combination groups (Cis200: cisplatin 200 µM; N1, N10, and N50: nimodipine 1, 10, and 50 µM, *p<0.05, **p<0.01, ***p<0.001)

		n	Mean±SE	Median (25%–75%)	p*	Pairwise comparisons
1	Cis200	72	0.591±0.017	0.619 (0.497–0.701)		
2	Cis200+N1	12	0.657±0.014	0.646 (0.626–0.698)		
3	Cis200+N10	47	0.676±0.014	0.696 (0.603–0.746)		1–5**, 1–6**,
4	Cis200+N50	24	0.658±0.030	0.680 (0.648–0.727)	<0.001	1–7**, 2–5*,
5	N1	8	0.167±0.040	0.177 (0.053–0.261)		3–6***, 4–7***
6	N10	11	0.206±0.060	0.120 (0.027–0.412)		
7	N50	18	0.272±0.032	0.270 (0.196–0.367)		

*Kruskal–Wallis one-way analysis of variance on ranks (median 25%–75%)

Table 3. Relative cell death values for the cisplatin/glibenclamide combination groups (Cis200: cisplatin 200 µM; G1, G10, and G50: glibenclamide 1, 10, and 50 µM, **p<0.01, ***p<0.001)

		n	Mean±SE	Median (25%–75%)	p*	Pairwise comparisons
1	Cis200	72	0.591±0.017	0.619 (0.497–0.701)		
2	Cis200+G1	12	0.695±0.020	0.703 (0.648–0.737)		
3	Cis200+G10	36	0.726±0.011	0.745 (0.685–0.769)		1–5**, 2–5***,
4	Cis200+G50	12	0.687±0.033	0.727 (0.590–0.770)	<0.001	1–7***, 4–7***,
5	G1	9	0.233±0.048	0.177 (0.100–0.388)		1–6**, 3–6***,
6	G10	12	0.296±0.048	0.276 (0.153–0.439)		1–3***
7	G50	23	0.274±0.038	0.231 (0.126–0.385)		

*Kruskal–Wallis one-way analysis of variance on ranks (median 25%–75%)

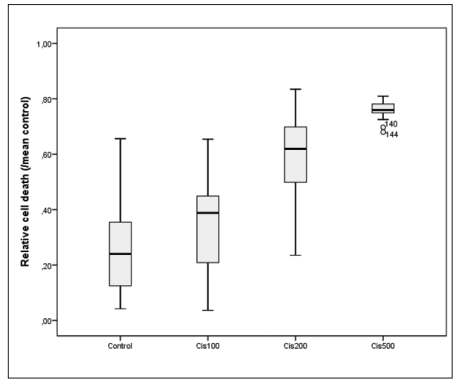

Figure 1. Cisplatin neurotoxicity at 100, 200, and 500 µM concentrations
cis: cisplatin

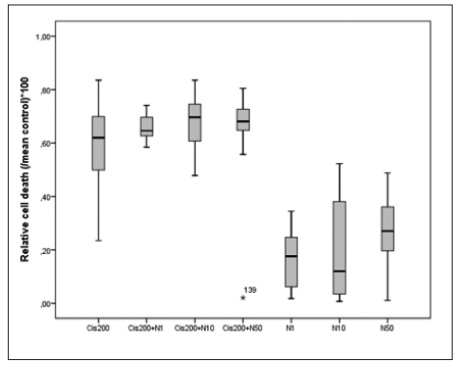

Figure 2. Cisplatin neurotoxicity at 100, 200, and 500 µM concentrations
cis: cisplatin

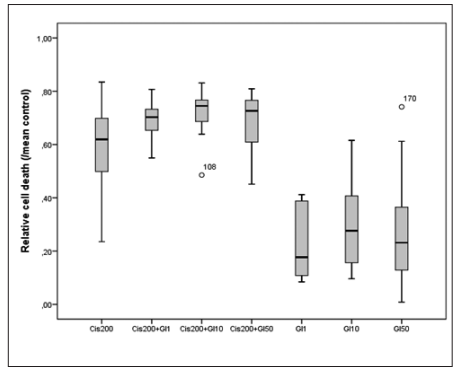

Figure 3. The relative cell death values of cisplatin and glibenclamide combination groups
cis: cisplatin, GI: glibenclamide

Na^+/Ca^{2+} exchanger) rather than L-type calcium channels as shown in a previous study [22].

L-type calcium channels were shown to couple with Ca^{2+}/CaM kinase II (CaMKII), enabling the channels to play a primary role in CaMKII activation [23]. The inhibition of CaMKII was shown to cause neurotoxicity via dysregulation of glutamate/calcium signaling and enhanced neuronal excitability [24]. CaMKII can also regulate cAMP response element-binding protein (CREB) phosphorylation, which is crucial for neuronal survival. Thus, blockade of L-type calcium channels with nimodipine may cause inactivation of CaMKII and disruption of CREB phosphorylation, leading to neurotoxicity and neuronal cell death [25]. Future studies should aim to investigate the roles of these molecules.

The physiology of calcium signaling also differs from cell to cell. Both Purkinje neurons and cerebellar granule neurons may have different signaling mechanisms [22]. Hence, unsuccessful inhibition of neurotoxicity by voltage-gated channels may have originated from their different calcium signaling mechanisms. However, previous studies have shown that nimodipine protects CGCs from intracellular calcium increase induced by NMDA and hydrogen sulfur neurotoxicity [26, 27]. In our previous study, we also studied CDDP neurotoxicity with different types of neuronal cells, including dorsal root ganglion neurons. CDDP also induced physiological alterations related to calcium dynamics in the cell. Nimodipine was able to protect the dorsal root ganglion neurons from the toxic effects of CDDP [28]. However, CGCs were different from the dorsal root ganglion neurons because the cerebellum is one of the regions of the central nervous system that develops postnatally [29]. CGC progenitors still migrate into the internal granular layer of the cerebellum even during early postnatal period [30]. Thus, at that time, CGCs undergo rapid growth and may be more vulnerable to the devastating effects of drugs. Because injury may generate some structural changes in this region. For example, Piccolini et al. [31] noticed that CDDP induces alterations in matrix metalloproteinase expression in the developing rat cerebellum. The information related to such physiological changes in CGCs in the presence of a neurotoxic drug CDDP is limited.

In addition, the capability of nimodipine to block channels can be based on the state of the channels, and channels bind nimodipine with different affinities according to their state [32]. The nimodipine-sensitive current is most probably responsible for the increase of calcium as determined by prolonged depolarizations in these cells. Thus, the sensitivity of voltage-gated calcium channels to nimodipine may be different based on the type of cells and condition.

In excitable cells, such as neurons, the regulation of potassium ion movements is fundamental for the regulation of membrane potential and electrical activity. Any change in the concentration of potassium ions that occurred in intracellular or intercellular compartments contributes to the activation of cell death mechanisms. K_{ATP} channels deliver potassium inside or outside of the cell according to the membrane potential. K_{ATP} channels decrease membrane excitability by regulating K^+ ion flow direction. Thus, they may be the candidate ion channels for the protection of cells from excitotoxicity [33].

At normal physiological status, K_{ATP} channels are closed; however, in the presence of a toxic agent, the membrane is depolarized [33]. This causes an excitation in the cells. K_{ATP} channels open, and K^+ ions flow through the outside of the membrane to defeat excitation and then hyperpolarization occurs. If K_{ATP} channels are exogenously closed, excitation cannot be blocked and excitotoxicity occurs in the cells.

In the study, to investigate the relationship between the neurotoxicity of CDDP and K^+ ion movement across the membrane through K_{ATP} channels, glibenclamide (blocker of K_{ATP} channel) at 1, 10, and 50 μM concentrations was applied with CDDP. Glibenclamide did not cause any significant change in CDDP neurotoxicity.

First, in the presence of a neurotoxic agent, K_{ATP} channels are opened to suppress excitation; however, later, the loss of excess potassium ion from the cell may contribute to the toxicity of CDDP. If the level of potassium decreases, some enzymes, such as caspases and endonucleases, may get activated, leading to induction of apoptosis [34].

Similarly the efflux of K^+ ions and the loss of intracellular potassium are also critical in apoptosis, like in case of Ca^{2+} influx and the accumulation of intracellular calcium [5]. Physiological concentrations of intracellular potassium act as a suppressor on proapoptotic molecules. If the loss of potassium is greater than the cell can tolerate, caspases, cytochrome C, and endonucleases would be activated, and then apoptotic cascade starts for these cells. Moreover, the cells lose water with potassium efflux, and this may cause shrinkage of the cells by decreasing cell volume, which is one of the characteristic features of apoptosis. Potassium ion is abundant in the cells, and even if tens of mM loss can be tolerated, a 50% loss cannot be tolerated by the cells. Thus, high potassium loss must be considered for causes of cell death. Therefore, the contribution of K_{ATP} channels to CDDP neurotoxicity was so complex. Mitochondrial K_{ATP} channels may be more effective to decrease toxicity as shown in a previous study [13]. K_{ATP} channels of neonatal rats in our study might be inadequate to protect the neurons from cell death. In an earlier study, Xia and Haddad showed that the formation of K_{ATP} channels and sulfonylurea receptors (subunit of K_{ATP} channels) occurs postnatally, and it is the highest level at maturation. Thus, K_{ATP} channel activation in the mature period may be more protective against the neurotoxic reaction [35].

The present study has some limitations. First, the content of the study was restricted to only one type of calcium and potassium channels. In addition, only the cytotoxicity test was performed to investigate the effects of drugs. MTT assay is an absorbance-based assay that provides information about the viability of the cells and how well they metabolize the component. It is not always comparable to % growth inhibition. In future studies, electron microscopy techniques may also be included in the study to detect the histopathological features of CDDP-induced neurotoxicity.

Finally, this preliminary study provides an insight for future studies in CDDP-induced neurotoxicity. It is difficult to explain the exact mechanisms of the interaction between the neurotoxicity of CDDP and calcium/potassium ion movements in CGCs. The results of the previous studies are also complicated to understand the missing points related to ionic homeostasis and neurotoxicity. The mechanisms of the interaction between the effects of CDDP and intracellular ionic homeostasis are complex and multidirectional phenomena that must be comprehensively investigated.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Eskişehir Osmangazi University (273/2012).

Informed Consent: Written informed consent was obtained from patients who participated in this study.

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