THE EFFECTS OF CISPLATIN AND ITS PT(II) ANALOGUE ON OXIDATIVE STRESS OF ISOLATED RAT HEART

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EFEKTI CISPLATINE I PT(II) ANALOGA CISPLATINE NA OKSIDACIONI STRES IZOLOVANOG SRCA PACOVA

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ABSTRACT

To date, numerous platinum (II) complexes have been successfully used in the treatment of different types of cancer. Therapeutic platinum complexes are different in terms of their structure, chemical reactivity, solubility, pharmacokinetics and toxicity. The aim of our research was the evaluation of cardiotoxicity of dichloro-(ethylendiamine) platinum (II) in a model of isolated rat heart using the Langedorff technique. Oxidative stress was assessed by determination of superoxide anion radical, hydrogen peroxide, Thiobarbituric Acid Reactive Substances and nitric oxide levels from coronary venous effluent. All reagents were perfused at increasing concentrations from 10^{-8} to 10^{-4} M for 30 minutes. In this paper, we report that substances administered at higher doses did not induce dose-dependent effects on oxidative stress markers. The results of this research may be of great interest for future studies in this area. There are many novel platinum compounds that had previously demonstrated antitumour activity, and these types of experiments in our study can assist in the examination of their cardiotoxicity. These results could be helpful for understanding dose-dependent side effects of existing and novel platinum compounds.

Keywords: *isolated rat heart, oxidative stress, novel-etilendiamin complex of platinum, cisplatin*

SAŽETAK

Danas se veliki broj kompleksa Pt(II) sa velikim uspehom koristi u terapiji različitih vrsta tumora. Kompleksi platine koji se koriste u terapijske svrhe se razlikuju u pogledu strukture, hemijske reaktivnosti, rastvorljivosti, farmakokinetičkih osobina i toksičnosti. Cilj ovog istraživanja bio je da se utvrdi kardiotoksičnost kompleksa dihloro-etilendiamin-platina(II) na modelu izolovanog srca pacova metodom po Langedorff-u. Oksidativni stres je određivan merenjem superoksid anjon radikala, vodonik peroksida, indeksa lipidne peroksidacije i azot-monoksida u koronarnom venskom efluentu. Sve supstance su aplikovane u rastućim dozama od 10^{-8} do 10^{-4}M tokom 30 minuta. U ovom radu smo naveli da ispitivane supstance pri višim dozama nisu pokazale dozno zavisni efekat na parametre oksidativnog stresa. Rezultati ovog istraživawa mogu biti od velikog značaja za neka nova istraživawa u ovoj oblasti. Postoji veliki broj novih kompleksa platine koji su pokazali antitumorsku aktivnost i ovakav vid eksperimenata bi mogao da posluži za ispitivawe njihovih kardiotoksičnih efekata. Ovi rezultati bi mogli biti od koristi za razumevanje dozno zavisnih neželjenih efekata postojećih i novih komplesa platine.

Ključne reči: *izolovano srce pacova, oksidativni stres, novi etilendiaminski kompleksi platine, cisplatina*

ABBREVIATIONS

DNA - deoxyribonucleic acid
EN – ethylenediamine
GSH - tripeptide gluthathione
H_2O_2 - hydrogen peroxide
$K_2[PtCl_4]$ - Potassium-tetra-chloroplatinum
NHCP - platinum-N-heterocyclic carbene complex
NO - nitric oxide
$O_2^{\cdot-}$ - superoxide anion radical
$Pt^{(II)}ENCl_2$ - **cis**-$[Pt(en)Cl_2]$ – dichloro-(ethylendiamine) platinum(II)
RNA - ribonucleic acid
ROS - reactive oxidative species
TBARS - Thiobarbituric Acid Reactive Substances

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INTRODUCTION

Synthesis of Peyron`s chloride (cisplatin) was the first step in research on biologically active platinum complexes; the biological activity of cisplatin was discovered many years later (1). To date, numerous platinum (II) complexes have been successfully used for the treatment of different types of cancer (2). Therapeutic platinum complexes differ in terms of their structure, chemical reactivity, solubility, pharmacokinetics and toxicity. Cisplatin affects tumour cells and prevents synthesis and repair of DNA (3). Nucleophiles such as DNA, RNA and proteins interact with cisplatin and cause several side effects (4, 5). Cisplatin has a central role in chemotherapy, but severe toxicity (e.g., nephrotoxicity, peripheral neuropathy, ototoxicity, neutropenia, thrombocytopenia, embryotoxicity, mutagenicity and cardiotoxicity) and cross-resistance have limited its therapeutic use and initiated development of new analogues (2, 6, 7).

Cisplatin-induced nephrotoxicity, ototoxicity and neurotoxicity have been elucidated in detail, but less is known about cardiotoxicity (8-11). The toxic effects of cisplatin may be caused by inhibition of protein synthesis, DNA damage, peroxidation of the cell membrane and mitochondrial dysfunction (12). One serious side effect of cisplatin treatment is acute, cumulative cardiotoxicity, which can be a limiting factor for its use in therapy. Cardiotoxicity resulting from the use of cisplatin is caused by the formation of reactive oxidative species (ROS) and the induction of immunogenic reactions by the presence of antigen presenting cells in the heart tissue (13). A few cases of acute myocardial infarction after treatment with cisplatin have been described previously (14, 15). Studies have shown that cisplatin stimulates the production of ROS such as superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), Thiobarbituric Acid Reactive Substances (TBARS), and nitric oxide (NO) (16).

The aim of our research was to evaluate cardiotoxicity by direct perfusion of isolated rat heart with cis-$[Pt(NH_3)_2Cl]$ and cis-$[Pt(en)Cl_2]$ ($Pt^{(II)}ENCl_2$). Oxidative stress was assessed by the measurement of $O_2^{\cdot-}$, H_2O_2, TBARS and NO levels in coronary venous effluent.

MATERIALS AND METHODS

Isolated rat heart preparation

Male Wistar-albino rats (body weight 180-200 g) were anaesthetized with diethyl ether and were killed by cervical dislocation according to Schedule 1 of the Animals (Scientific procedures) Act 1986, UK. After urgent thoracotomy and rapid arrest of the beating heart by superfusion with ice-cold isotonic saline, the hearts were isolated and perfused according to the Langendorff technique. The composition of the Krebs-Henseleit buffer (perfusion medium) was as follows (in mmol/l): NaCl (118); KCl (4.7); $CaCl_2 \times 2H_2O$ (2.5); $MgSO_4 \times 7H_2O$ (1.7); $NaHCO_3$ (25); KH_2PO_4 (1.2); glucose (5.5). The buffer was equilibrated with a gas mixture (5% CO_2-95% O_2) at 37°C (pH 7.4). Constant left ventricular draining through the dissected mitral valve was performed, and the sensor (*transducer BS4 73-0184, Experimetria Ltd, Budapest, Hungary*) was inserted into the left ventricular cavity via the left atrium for continuous recording of the functional cardiac parameters (17).

Perfusion of isolated rat heart

After 30 minutes of stabilization, myocardial perfusion was established at a constant coronary perfusion pressure of 70 cm H_2O. Once flow was stabilized, hearts were perfused for 30 minutes with Krebs-Henseleit buffer without (control group) or with the following test compounds at different concentrations from 10^{-4} to 10^{-8} M: $Pt^{(II)}ENCl_2$, cisplatin, potassium-tetra-chloroplatinum ($K_2[PtCl_4]$), ethylenediamine (EN) and Krebs-Henseleit buffer.

Biochemical assays

Samples of coronary venous effluent were collected at the end of each period of perfusion (30, 60, 90, 120 minutes).

Index of lipid peroxidation
(Thiobarbituric Acid Reactive Substances – TBARS)

The degree of lipid peroxidation in coronary venous effluent was estimated by measuring thiobarbituric acid reactive substances (TBARS). The measurement was taken using 1 % thiobarbituric acid (TBA) in 0.05 sodium hydroxide (NaOH) and was incubated with coronary effluent at 100 °C for 15 minutes. The results were read at 530 nm. Krebs-Hensenleit solution was used as a blank probe (18).

Nitrite determination

Nitric oxide was assessed as nitrite and quantified by the spectrophometric method using the *Griess* reagent. 0.5 ml of perfusate was precipitated with 200 µl of 30% sulfosalicylic acid, vortexed for 30 min and centrifuged at 3000 x g. Equal volumes of the supernatant and Griess reagent, containing 1% sulphanilamide in 5% phosphoric acid and 0.1% naphthalene ethylenediamine-dihydrochloride, were added and incubated for 10 min in the dark. The results were read at 543 nmol/l. Nitrite levels were calculated using sodium nitrite as a standard (19).

Superoxide determination

The level of superoxide anion radical ($O_2^{\cdot-}$) in coronary venous effluent was measured using a Nitro Blue Tetrazolium (NBT) reaction in TRIS-buffer. The results were read at 530 nm. Krebs-Hensenleit solution was used as a blank probe (20).

Hydrogen peroxide determination

The level of hydrogen peroxide (H_2O_2) in coronary venous effluent was determined by measuring phenol red oxidation of H_2O_2 in the presence of horse-radish peroxidase. The results were read at 610 nm (21).

Reagents

$Pt^{(II)}ENCl_2$ was synthesized according to Keppler et al (22). Cisplatin, $K_2[PtCl_4]$, and EN as well as substances

necessary for the preparation of Krebs-Henseleit buffer were purchased from Sigma-Aldrich GmbH, Germany.

Statistical Analysis

The concentration-response relationship was determined by linear regression on logarithmically transformed data calculated according to the least squares method. The effect of each concentration of tested substances was expressed as a percentage of the maximal response. Significance of the linear regression was tested by analysis of variance, with a p-value of less than 0.05 considered significant. For each substance, the concentration that gave 50% of the maximum response (EC_{50}) was calculated.

RESULTS

Superoxide anion radical ($O_2^{\cdot-}$)

Superoxide anion radical ($O_2^{\cdot-}$) levels were not significantly affected by treatment with $Pt^{(II)}ENCl_2$ (from 10^{-4} M to 10^{-8} M; F=1.72, df_1=4, df_2=25, p>0.05), EN (from 10^{-4} M to 10^{-8} M; F=1.21, df_1=4, df_2=25, p>0.05), $K_2[PtCl_4]$ (from 10^{-4} M to 10^{-8} M; F=0.73, df_1=4, df_2=25, p>0.05) and cisplatin (from 10^{-4} M to 10^{-8} M; F=1.02, df_1=4, df_2=25, p>0.05).

Thiobarbituric Acid Reactive Substances (TBARS)

Thiobarbituric Acid Reactive Substances (TBARS) levels were not significantly affected by treatment with $Pt^{(II)}ENCl_2$ (from 10^{-4} M to 10^{-8} M; F=0.14, df_1=4, df_2=25, p>0.05), EN (from 10^{-4} M to 10^{-8} M; F=0.53, df_1=4, df_2=25, p>0.05), $K_2[PtCl_4]$ (from 10^{-4} M to 10^{-8} M; F=0.15, df_1=4, df_2=25, p>0.05) and cisplatin (from 10^{-4} M to 10^{-8} M; F=1.06, df_1=4, df_2=25, p>0.05).

Nitric oxide (NO)

Nitric oxide (NO) levels were not significantly affected by treatment with $Pt^{(II)}ENCl_2$ (from 10^{-4} M to 10^{-8} M; F=0.97, df_1=4, df_2=25, p>0.05), EN (from 10^{-4} M to 10^{-8} M; F=1.13, df_1=4, df_2=25, p>0.05), $K_2[PtCl_4]$ (from 10^{-4} M to 10^{-8} M; F=1.19, df_1=4, df_2=25, p>0.05) and cisplatin (from 10^{-4} M to 10^{-8} M; F=0.75, df_1=4, df_2=25, p>0.05).

Hydrogen peroxide (H_2O_2)

Hydrogen peroxide (H_2O_2) levels were not significantly affected by treatment with $Pt^{(II)}ENCl_2$ (from 10^{-4} M to 10^{-8} M; F=0.14, df_1=4, df_2=25, p>0.05), EN (from 10^{-4} M to 10^{-8} M; F=0.26, df_1=4, df_2=25, p>0.05), $K_2[PtCl_4]$ (from 10^{-4} M to 10^{-8} M; F=0.82, df_1=4, df_2=25, p>0.05) and cisplatin (from 10^{-4} M to 10^{-8} M; F=0.81, df_1=4, df_2=25, p>0.05).

DISCUSSION

Cisplatin use leads to acute and cumulative cardiovascular complications such as arrhythmias, myocarditis, cardiomyopathy and electrocardiographic changes (23). These complications have led to the reduction of cisplatin doses or the discontinued use of cisplatin for chemotherapy (24). Drug-induced oxidative stress is generally one of the key features involved in the mechanism of toxicity in the cardiovascular system. It is well known that most anticancer drugs are associated with toxic side effects and that the formation of ROS plays a crucial role in the mechanism of anticancer drug-induced cardiotoxicity. There is no clear evidence for the cellular and molecular mechanisms involved in cisplatin cardiotoxicity, but some experimental and clinical studies support the idea that an increase in oxidative stress may lead to cardiotoxicity (24-28).

Cisplatin is a potent chemotherapeutic agent that exhibits multiorgan toxicity. *In vitro* studies have shown that ROS such as $O_2^{\cdot-}$, H_2O_2 and ·OH are involved in the cytotoxicity induced by cisplatin. The role of oxidative stress in the pathophysiology of cisplatin-induced toxicity was investigated by using different antioxidants and superoxide dismutase mimetics and it was shown that the use of ROS scavengers prevents or reduces cisplatin-induced cytotoxicity. These results provide additional evidence that ROS have important roles in the pathogenesis of cytotoxicity induced by cisplatin (12, 27, 29). There is evidence that acute administration of cisplatin leads to a significant increase in the biochemical markers of oxidative stress in postmitochondrial and mitochondrial fractions in cardiac tissues in rats (30).

Lower doses of cisplatin induce apoptosis mediated by superoxide and hydroxyl radicals, and higher doses of cisplatin induce necrosis mediated by superoxide and hydrogen peroxide in renal tubular epithelial cells (29, 31-33). In our research, we applied two different platinum complexes at increasing concentrations (range 10^{-8}-10^{-4}) to isolated rat heart. As seen in Tables 1 and 2 , when the hearts were exposed to lower concentrations of the complexes (range 10^{-8}-10^{-7}), the production of oxygen free radicals was higher than when the hearts were exposed to higher concentrations. This phenomenon has been previously observed as the mechanism of cisplatin cytotoxicity that is responsible for cell death in culture (12).

In addition to the production of oxygen free radicals, there is evidence that cisplatin induces lipid peroxidation and decreases the activities of antioxidant enzymes in rat kidneys (34). Cisplatin especially decreases the activity of the tripeptide gluthathione (GSH), which represents its most important non-DNA target (35). GSH is known to protect mitochondria against oxidative stress, inhibiting free radical mediated injury by eliminating hydrogen peroxides. Oxidation of GSH by cisplatin changes the intramitochondrial redox status, and contributes to the establishment of a prooxidative state that favours generation of hydroxyl radicals and oxidative damage to macromolecules such as mitochondrial proteins and lipids. The depletion of GSH seems to be one of the most important factors for lipid peroxidation (36). In our research, we measured TBARS as an index of lipid peroxidation (Table 3), and we noticed that cisplatin induced higher lipid peroxidation than $Pt^{(II)}ENCl_2$ (concentration range 10^{-8}-10^{-6}); However, both substances caused a decrease in TBARS levels at the

Table 1. The effects of cisplatin, $Pt^{(II)}ENCl_2$, EN, K_2PtCl_4 on H_2O_2

	X±SD (nmol/l)				
	Control	Cisplatin	$Pt^{(II)}ENCl_2$	EN	K_2PtCl_4
10^{-8}	23.54 ± 13.26	13.61 ± 4.33	30.72 ± 8.00	7.84 ± 5.22	2.97 ± 2.74
10^{-7}	18.58 ± 5.43	11.90 ± 6.50	26.27 ± 15.93	7.77 ± 8.28	1.40 ± 1.25
10^{-6}	19.67 ± 7.48	9.28 ± 5.03	18.43 ± 11.57	8.39 ± 6.82	1.08 ± 0.53
10^{-5}	17.30 ± 10.94	8.95 ± 5.30	15.48 ± 9.96	3.68 ± 2.56	0.55 ± 0.51
10^{-4}	20.01 ± 14.97	4.47 ± 2.96	14.10 ± 8.13	0.53 ± 0.51	0.26 ± 0.20

Table 2. The effects of cisplatin, $Pt^{(II)}ENCl_2$, EN, K_2PtCl_4 on $O_2^{\cdot-}$

	X±SD (nmol/l)				
	Control	Cisplatin	$Pt^{(II)}ENCl_2$	EN	K_2PtCl_4
10^{-8}	55.72 ± 18.09	23.80 ± 14.61	32.73 ± 24.29	26.06 ± 12.31	36.40 ± 22.90
10^{-7}	73.52 ± 40.52	43.96 ± 22.35	35.19 ± 29.48	27.57 ± 11.16	29.16 ± 18.95
10^{-6}	45.04 ± 26.20	27.93 ± 26.95	12.99 ± 5.93	18.80 ± 12.46	52.90 ± 47.37
10^{-5}	70.17 ± 42.31	14.36 ± 8.84	28.21 ± 18.51	30.85 ± 18.85	21.47 ± 15.83
10^{-4}	49.87 ± 15.36	13.14 ± 12.36	19.98 ± 7.67	2.20 ± 1.48	8.95 ± 5.16

Table 3. The effects of cisplatin, $Pt^{(II)}ENCl_2$, EN, K_2PtCl_4 on TBARS

	X±SD (nmol/l)				
	Control	Cisplatin	$Pt^{(II)}ENCl_2$	EN	K_2PtCl_4
10^{-8}	14.72 ± 9.91	28.68 ± 23.76	15.57 ± 4.84	12.51 ± 6.02	40.04 ± 18.38
10^{-7}	11.73 ± 10.26	20.65 ± 16.24	13.81 ± 8.57	10.20 ± 7.33	48.84 ± 18.59
10^{-6}	14.95 ± 10.50	24.56 ± 11.41	12.76 ± 9.37	16.52 ± 11.83	37.10 ± 22.36
10^{-5}	12.58 ± 9.23	15.97 ± 10.15	10.41 ± 9.73	12.10 ± 4.09	18.35 ± 6.52
10^{-4}	13.56 ± 8.10	8.49 ± 4.60	5.23 ± 5.23	6.95 ± 3.43	7.03 ± 4.39

Table 4. The effects of cisplatin, $Pt^{(II)}ENCl_2$, EN, K_2PtCl_4 on NO

	X±SD (nmol/l)				
	Control	Cisplatin	$Pt^{(II)}ENCl_2$	EN	K_2PtCl_4
10^{-8}	5.20 ± 4.39	10.24 ± 4.79	15.13 ± 6.88	3.62 ± 1.80	12.19 ± 3.96
10^{-7}	3.72 ± 2.40	10.60 ± 4.20	6.16 ± 6.98	2.60 ± 1.53	8.90 ± 3.90
10^{-6}	2.35 ± 1.94	8.99 ± 2.22	3.71 ± 4.65	3.36 ± 1.36	7.58 ± 1.12
10^{-5}	2.80 ± 1.33	6.29 ± 4.16	4.67 ± 4.16	3.21 ± 1.80	3.81 ± 0.62
10^{-4}	2.05 ± 1.46	3.12 ± 0.85	1.56 ± 1.12	0.24 ± 0.21	1.60 ± 0.82

higher concentration range (10^{-5}-10^{-4}), possibly because of myocardium necrosis.

Administration of cisplatin causes overproduction of nitric oxide in the heart and kidney. Overproduction of NO may increase cellular injury by decreasing intracellular GSH levels and production of peroxynitrite anion, which causes protein nitration and tissue injury (31). In our research, we measured NO as a parameter of nitrosative stress (Table 4), and it can be observed that production of NO is higher at lower doses of each complex (concentration range 10^{-8}-10^{-7}) and is lower at higher doses (concentration range 10^{-6}-10^{-4}).

Many scientists have studied the connection between cisplatin use and imbalance of production and removal of

ROS. They observed the effects of joint use of cisplatin and different antioxidants on production of ROS in various tissues such as liver, heart and kidney. They showed that acute administration of cisplatin to rats (a single dose of 5-30 mg/kg) leads to overproduction of oxidative stress and reduction of antioxidant defences. When cisplatin was administered to rats together with certain antioxidants, the production of ROS was lower in liver, heart and kidney tissues (29-31, 37-38).

In accordance with the potential of cisplatin to cause damage to various tissues because of oxidative stress, there have been attempts to overcome these side effects. One such attempt was the formulation of new generations of platinum complexes (1). Ciftci et al. tried to prove that novel platinum agents, such as the platinum-N-heterocyclic carbene complex (NHCP), are less toxic than cisplatin (39). This study had the same design as earlier studies (30, 32, 36). The researchers showed that NHCP produced the same oxidative stress level as cisplatin at the lower dose of 5 $mgkg^{-1}$, but at the higher dose of 10 $mgkg^{-1}$, NHCP was more toxic than cisplatin.

In our study, we investigated the influence of acute administration of $Pt^{(II)}ENCl_2$ (concentration range: 10^{-8}-10^{-4}) to isolated rat heart compared to treatment with cisplatin (same concentration range) on oxidative stress parameters (Tables 1-4). We also tried to prove that $Pt^{(II)}ENCl_2$ is less toxic than cisplatin. However, our results show that, regardless of the dose, neither platinum complex induced statistically significant changes in redox status.

The results of this research may be of great interest for future studies in this area. There are many novel platinum compounds that had previously been shown to exhibit antitumour activity, and the types of experiments in our study could assist in the examination of their cardiotoxicity. Our results could be helpful for understanding dose-dependent side effects of existing and novel platinum compounds.

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