THE POSSIBILITY ROLE OF INTERLEUKINS 6 AND 17 IN CISPLATIN-INDUCED STRUCTURAL CHANGES WITHIN THE CEREBELLAR CORTEX OF DEVELOPING ALBINO RATS

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ABSTRACT:
The cisplatin (CisPt)-induced central neurotoxicity and its possible mechanisms have always been under the scope, particularly during development, but few studies are available. As a result, the goal of this study was to see if there were any changes in interleukins 6 and 17 (IL-6 and IL17) levels in the postnatal rat cerebellum, as well as any associated histological abnormalities, after CisPt treatment. Forty newborn pups were divided into two equal experimental groups: the control group, which was kept without any treatment, and the CisPt-treated group that received a single subcutaneous injection of CisPt (5 µg/g b.w.) in their nape at PD10. Ten rats at PD17 and PD30 ages were deeply anesthetized and sacrificed from each group. CisPt-treated rats showed a statistically significant increase of IL-17 at PD17 and PD30, while IL-6 revealed a statistically significant increase at PD17. Histopathologically, CisPt caused congestion in the blood vessels at PD17 and profound degenerative changes within the Purkinje cells at PD17 and PD30. In conclusion, CisPt caused detrimental effects on the structure of the developing cerebellar cortex that may be inflammatory-related.

Key words: Cisplatin, Cerebellum, interleukins, Postnatal-rats

I. INTRODUCTIONS:
Cisplatin is a well-known widely used platinum-based chemotherapeutic agent (Chiorazzi et al., 2015). Platinum compounds are included in many pediatric and adult oncologic treatment protocols (Stathopoulos, 2010). Breast, testicular, prostate, stomach, ovarian, esophageal cancers, mesothelioma, melanoma, and other oncologic and hematologic malignancies are all treated with cisplatin alone or in combination with other chemotherapeutic drugs or even radiotherapy (Crona et al., 2017). Unfortunately, the higher cumulative CisPt doses cause ototoxicity (Brock et al., 2012), cardiotoxicity (Chowdhury et al., 2016), nephrotoxicity (Li et al., 2017), and hepatotoxicity (Pezeshki et al., 2017).

Among the platinum compounds, CisPt is the most neurotoxic (Amptoulach & Tsavaris, 2011). It has a wide range of neurotoxicity including peripheral neuropathy, vestibulopathy, encephalopathy, cerebellar syndrome, and cognitive impairment (Dietrich et al., 2006; Vargo et al., 2017). Furthermore, the cerebellum, particularly the cerebellar cortex and Purkinje neurons, is very sensitive to intoxication and poisoning (Manto, 2012).

The precise mechanism of CisPt-induced neurotoxicity is still unknown (Kanat et al., 2017). Cisplatin or its metabolites have been shown to interact with a range of cellular organelles, including nucleus, cell membranes, lysosomes, and endoplasmic reticulum, causing cellular necrosis and apoptosis (Gatti et al., 2015). Oxidative damage, inflammation, mitochondrial malfunction, and DNA damage are some of the other pathophysiological pathways of CisPt-induced neurotoxicity (England et al., 2013). Furthermore, oxidative stress and inflammation could
be linked in the development of CisPt-induced neurotoxicity. Overproduction of ROS caused by CisPt has been discovered to speed up the release of pro-inflammatory cytokines (Akman et al., 2015).

The production and release of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, IL-17, and IL-18 by activated cells such as neurons, astrocytes, microglia, and endothelial cells characterize the inflammatory response during cerebral injury (Siniscalchi et al., 2014). IL-6 is a pleiotropic cytokine that functions in various biological systems and is abundantly expressed in the central nervous system (CNS) (Kang et al., 2019). IL-17 is a prototype member of the IL-17 family of cytokines, which contains six structurally related isoforms: IL-17A-F (Chen & O'Shea, 2008). This is the first study to look at how CisPt therapy impacts IL-6 and IL-17 levels in the postnatal cerebellum, as far as we know. Such knowledge, however, will be critical not only in estimating the role of IL-6 and IL-17 in the pathogenesis of CisPt-induced neurotoxicity but also in developing new therapeutics for patients to alleviate its deleterious effects on the CNS.

II. MATERIAL AND METHODS:

Drugs:

Cisplatin (CisPt): 1mg/ml, Mylan Pharmaceuticals was used. It was administrated by subcutaneous injection (Sc) in the nape of the neck at a dose of 5 μg/g body weight (b.w.) on the basis of the dose suggested by previous literature (Bodenner et al., 1986). This dose was corresponding to the therapeutic dose of CisPt.

III. METHODS:

Experimental animals:

The study started with mature albino rats (8 females and 4 males). All rats were kept individually in an animal room with a 12/12 h light-dark cycle, a temperature range of 20°C to 22°C, and a normal relative humidity. After 1-week adaptation, adult males were housed with adult females at a ratio of 1:2 respectively in each cage for mating. To detect the occurrence of pregnancy, the vaginal smear was taken from the females on the next day of mating. Day one of gestation was calculated if sperms presented in the vaginal smear. For approximately 21 days of gestation, the pregnant female rats were kept in individual plastic cages.

Newborn pups were housed in cages with their mothers for breastfeeding. Forty infantile male pups [at postnatal day (PD)10] were divided into 2 groups: control (n = 20) and CisPt-treated (n = 20) groups. Ten rats were added to the treated group as a reserve and were bred at the same environmental conditions as other experimental rats used in this study. All experimental procedures were performed following the guidelines of the Institutional Animal Care and Use Committee (ZU-IACUC) of Zagazig University with Approval number; ZU-IACUC/3/F/35/2018.

Experimental designs:

For histological study and tissue homogenate assessment: forty rats were used and divided into two groups:

a. **Group A:** control groups (20 rats, 10 per stage): The rat pups were divided according to the day of scarification into two subgroups as follows: PD17, and PD30 control groups. Each subgroup was kept without any treatment all over the experimental duration.

b. **Group B:** Cisplatin (CisPt) treated groups (20 rats, 10 per stage): The rat pups at PD10 received a single dose of CisPt (5 μg/g b.w.) by subcutaneous injection in their nape. The CisPt-treated group was also divided according to the day of scarification into two subgroups as follows: PD17 and PD30 CisPt-treated groups.

Just before sacrificing the animals, they were anesthetized using sodium thiopental (75 mg/kg intraperitoneal injection [i.p]) (IACUC, 2013), then:

1. **For biochemical assays:** After opening the cranial cavity and removing the brain, the cerebella of the treated and control rats from the same age groups were dissected from the cerebra on an ice-cold surface, rinsed in PBS (pH 7.4) to remove excess blood thoroughly, dried, subsequently stored at -80°C for consequent homogenization and biochemical assessment of IL-6 and IL-17.

2. **For the histological specimens:** the thoracic cage was opened with perfusion of intracardiac saline through the left ventricle followed by formalin 10% to achieve perfect distribution of the fixative to every part of the tissue organs until paleness of the liver occurred as an indicator of good perfusion. Then, after the cranial cavity
was opened; the brain was carefully dissected out and left immersed in the buffered formalin fixative and undisturbed for one hour. Then the cerebella were separated from the brain for further histological preparation.

**Biochemical studies:**

The cerebella were homogenized in cold 50 mM sodium phosphate buffer (pH 7.4) containing 0.1 mM EDTA to give 5% homogenate (w/v). Afterwards, centrifugation of the homogenates was done at 2000-3000 rpm for 20 min at 4°C to remove nuclei and debris. The supernatants were separated, aliquoted, and stored at -80°C till subsequent chemical evaluation.

ELISA kits for rats (BT-laboratory, Shanghai) were used for determining interleukin-6 (IL-6, Catalog No: E0135Ra) and interleukin-17 (IL-17, Catalog No: E0115Ra) levels in the cerebellar tissues. The analysis was performed in line with the manufacturer's protocol for the commercial kits. The levels of IL-6 and IL-17 were calculated from the corresponding standard curves and expressed as ng/ml and pg/ml, respectively.

**Histopathological analysis:**

**Hematoxylin and eosin (H&E) staining:**

All steps were performed at the Histology and Cell Biology Department, Faculty of Medicine, Zagazig University. Cerebellum specimens were fixed in 10% neutral buffered formalin and embedded in paraffin, according to Bancroft & Layton, (2018). Sections of 5 μm thickness were mounted on glass slides, deparaffinized in xylene, and stained with hematoxylin and eosin (H&E). Stained slides were examined by light microscopy (The Leica DM500, Leica ICC50 W Camera Module) at the Image Analysis Unit of the Anatomy and Embryology Department, Faculty of medicine, Zagazig University.

**Statistical analysis:**

Statistical calculations were carried out using SPSS version 16.0 (Stehlik-Barry & Babinec, 2017). Since the data displayed normal distribution (parametric), continuous variables were expressed as mean ± standard deviation (SD). Normality was checked by Kolmogorov–Smirnov test. Independent T-test was used to detect significant differences between groups. Differences were considered statistically significant (*) at P-value < 0.05.

**Results:**

**Evaluation of IL-6 and IL-17 levels:**

The results of the mean values of IL-6 levels within the CisPt-treated rats revealed a significant statistical increase (P <0.05) within the PD17 age group and a non-significant increase at the PD30 age group when compared to the age-matched control groups (Table 1).

Statistical analysis of the mean values of IL-17 showed a significant statistical increase (P <0.05) within the PD17 and PD30 CisPt-treated age groups when compared with their counterpart ages of the control groups (Table 1).

The IL-6 and IL-17 in the PD17, and PD30 CisPt-treated groups showed a progressive decrease of their levels as the age progress in relation to the control groups of the same age i.e., IL-6 (1.56 then 1.44 folds) and IL-17 (2.35 then 1.57 folds) (Table 1).

**Table 1: Statistical assessment of the mean values of IL-6 and IL-17 using independent T-test.**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Age group</th>
<th>¹control</th>
<th>² CisPt -treated</th>
<th>t</th>
<th>P value (¹ vs ²)</th>
<th>treated / Control %</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 ng/ml</td>
<td>PD17</td>
<td>9.81 ± 2.5</td>
<td>15.34 ± 1.02</td>
<td>-3.541</td>
<td>0.024*</td>
<td>156.37%</td>
</tr>
<tr>
<td></td>
<td>PD30</td>
<td>8.75 ± 0.29</td>
<td>12.63 ± 1.85</td>
<td>-2.805</td>
<td>0.067 Ns</td>
<td>144.34%</td>
</tr>
<tr>
<td>IL-17 Pg/ml</td>
<td>PD17</td>
<td>68.19 ± 3.59</td>
<td>160.13 ± 37</td>
<td>-4.284</td>
<td>0.049*</td>
<td>234.83%</td>
</tr>
<tr>
<td></td>
<td>PD30</td>
<td>103.36 ± 18.55</td>
<td>162.15 ± 16.68</td>
<td>-3.612</td>
<td>0.034*</td>
<td>156.88%</td>
</tr>
</tbody>
</table>

N=6 for each subgroup

*: Significant (p<0.05) Ns: non-significant significant (p > 0.05).
IL-6: Interleukin-6  IL-17: Interleukin-17

Histopathological analysis:

Histological examination of the cerebellar sections using hematoxylin and eosin staining:
The cerebellar cortex of PD17 rat was formed of four layers [external granular (EGL), molecular (ML), Purkinje (PL), and internal granular (IGL) layers] (Figs. 1), but that of PD30 was formed of three layers (ML, PL, and IGL) (Figs. 2).

In the PD17 CisPt-treated group, the EGL was interrupted by some small vacuoles and the ML showed edematous neuropil. The PL showed profound degenerative effects with distorted, irregularly-shaped, and darkly-stained cells containing darkly stained nuclei. In addition, vacuolations appeared within the PL and the upper part of the IGL. The IGL revealed dilated congested blood vessels with wide peri-vascular spaces (Fig. 1).

Moreover, within the PD30 CisPt-treated group, most of the Purkinje cells were severely degenerated with dense shrunken nuclei and surrounded with pericellular spaces (Fig. 2), while some cells appeared with vesicular nuclei.

Figure 1 (a-b): Representative photomicrographs of H&E-stained sections of the cerebellar cortex of PD17 experimental rats. [a]: Cerebellar sections of control group showing thin external granular layer (EGL) with deeply stained granular cells. Few scattered, elongated, and vertically oriented migrating cells (M) appear within the molecular cell layer (ML). Purkinje cell layer (PL) exhibits Purkinje cells (P) with a large rounded vesicular nucleus (N). The internal granular layer (IGL) contains a huge number of small deeply stained granular cells separated by cerebellar islands (Star). [b]: Cerebellar sections of CisPt-treated group showing dilated congested meningeal blood vessel (asterisk *) and vacuolations (Arrowheads) within the EGL. The ML shows edematous neuropil. The PL contains irregular-shaped (Red P*) and deeply stained Purkinje cells with darkly stained nucleus (Black P*). The PL and upper part of the IGL shows many vacuolations (Thin arrow) in addition to congested blood vessels (BV) surrounded by wide perivascular (H&E × 400).
Figure 2 (a-b): Representative photomicrographs of H&E-stained sections of the cerebellar cortex of PD30 experimental rats. [a]: Cerebellar sections of the control group showing the outer molecular cell layer (ML) containing few scattered outer stellate cells (St) and deeper basket cells (B). The intermediate Purkinje cell layer (PL) contains flask-shaped Purkinje cells (P) with rounded vesicular nucleus (N). Their dendrites extend throughout the ML as a branching basket of nerve fibers (Red arrows). The internal granular layer (IGL) contains cerebellar islands (Star) intervening between granular cells. [b]: Cerebellar sections of CisPt-treated group showing degenerated deeply stained Purkinje cells (Black P*) surrounded with pericellular spaces (Thin black arrow) in the PL with other purkinje cells (Red P*) with vesicular nucleus. The ML appears with diminished thickness (H&E × 400).

IV. DISCUSSION:

Cisplatin is a widely used for the treatment of several tumors. Neurotoxicity has been identified as one of the most serious problems associated with CisPt chemotherapy (McWhinney et al., 2009). The laboratory rat is an essential part of today’s biomedical research. It is a well-known model in numerous fields, including neurobehavioral, cancer, and toxicological studies. Animal models are a useful tool for understanding human disease as well as basic biology (Schofield et al., 2012; Sengupta, 2013). Despite the fact that the cerebellar origins are determined early in embryogenesis, its maturation continues until postnatal life, making it vulnerable to abnormalities (Stevenson & Hall, 2006).

The light microscopic examination of the H&E stained sections of the present study verified that the cerebellar cortex of 17th days old albino rat is formed of four layers (external granular, molecular, Purkinje, internal granular), but that of 30 days old rats is formed of three layers (molecular, Purkinje, granular). At the 30th-day-old control group, the external granular layer was not present. This absence may be due to the fact that after PD15 the mitotic activity of the granule cell precursor (GCP) ceases and postmitotic granule cells migrate along the Bergmann glial processes towards the IGL. Migration is completed at 2 and 3 weeks after birth in mice and rats, respectively (Carletti & Rossi, 2008; Tanaka, 2009).

Following exposure to CisPt, the cerebellar cortex suffered from severe destructive effects. In the PD17 CisPt-treated group, the presence of vacuolations within the molecular, Purkinje, and granular cell layers was the hallmark. In addition, the Purkinje cell layer contained irregularly shaped and deeply stained cells with darkly stained nuclei, and the IGL showed congested blood vessels. Within the PD30 CisPt-treated group, the Purkinje cells were the most affected with being severely degeneration and surrounded by pericellular spaces with few cells possessing vesicular nuclei.
Our previous results go on hand with the adult study of Kandeil et al. (2019) who administrated 2 mg/kg of CisPt twice a week i.p. (a total of nine injections) and showed congested blood vessels and prominent oedema, with degenerative Purkinje cells within the cerebellar cortex. Vacuolations have been linked to altered lipid metabolism, sequestration of absorbed material, autophagy, endoplasmic reticulum stress, and proteasome dysfunction, according to Minaugh et al. (2006) and Franco and Cidlowski (2009).

Inflammation is becoming more widely recognized as a significant factor to CNS injury in both the developing and adult brain (Deverman & Patterson, 2009; Spann et al., 2018). Neuro-inflammation may be another potential mechanism in CisPt-induced toxicity in the developing cerebellum of rats. Interleukin-6 (IL-6) is a pro-inflammatory cytokine that affects brain functions, tissue regeneration, and metabolism and plays a vital role in cell proliferation and differentiation in humans. It regulates the production of several proteins involved in acute inflammation and infection (Scheller et al., 2011; Uciechowski & Dempke, 2020). Excessive IL-6 production and deregulation of IL-6 receptor signaling, on the other hand, are involved in disease pathogenesis (Kang et al., 2019).

In the present study, IL-6 levels within the CisPt-treated rats revealed a significant statistical increase within the PD17 age group and a non-significant increase at the PD30 age group when compared to the age-matched control groups. Our findings were in line with the adult study of Abdel-Wahab and Moussa (2019) on the rat brain with a single dose of 8 mg/kg CisPt intraperitoneally. Also, in agreement with adult rat studies on other organs as those of Shalkami et al. (2018) and Kandemir et al. (2019) on the kidney with a single dose of CisPt (7.5 mg/kg, i.p.) and the cardiac study of Chowdhury et al. (2016) who used CisPt at a dose of 10 mg/kg, i.p. for a week. On the other hand, some studies in mice found no evidence of an inflammatory response in the brain, as measured by IL-6 expression in addition to IL-1β and TNF-α as Chiu et al. (2017) who administrated CisPt at a dose of 2.5 mg/kg, i.p. for 2 cycles consisting of 5 daily injections with 5 days interval without injections.

IL-6 is upregulated in several animal models of brain injury and has a variety of functions (Ert et al., 2012), as evidenced by studies in IL-6 mutant mice, which have a hampered inflammatory response, increased oxidative stress, impaired neuroglial activation, reduced lymphocyte recruitment, and a slower rate of recovery and healing (Penkowa et al., 2000; Penkowa et al., 1999; Swartz et al., 2001).

Regarding cerebellar levels of IL-17 in the current study, there was a significant statistical increase within the PD17 and PD30 CisPt-treated age groups in relation to the control groups of the same age. IL-17 is a potent pro-inflammatory factor with an important role in inflammation (Korn et al., 2009). It mediates the pro-inflammatory responses via the induction of many other cytokines, including IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1β, TGF-β, TNF-α and chemokines, including IL-8 from many cells (Linden et al., 2005).

The human brain endothelium produces IL-17, which has been demonstrated to compromise the blood brain barrier (BBB)'s integrity (Rahman et al., 2018). This increased permeability of the BBB facilitates the CNS inflammation resulting in the recall of more CD4+ cells (Cipollini et al., 2019). Furthermore, according to Huppert et al. (2010), IL17 can disrupt the BBB by boosting superoxide generation, and the consequent excessive oxidative stress affects the endothelial cytoskeleton, resulting in further BBB dysfunction. IL-17 is also involved in neurovascular dysfunction, according to Nguyen et al. (2013).

Siffrin et al. (2010) suggested that Th17 cells (cells producing IL-17) interact directly with neurons forming antigen-independent immune synapse-like contacts. These neuron–Th17 cell contacts leads to increased intracellular calcium (Ca^{2+}), resulting in Ca^{2+} overload and neuronal damage.

Zimmermann et al. (2013) found that IL-17 expression alone might activate glial cells and promote neuroinflammatory responses and Waisman et al. (2015) linked the presence of IL-17 to astrocytes and microglia activation, as well as the consequences for disease progression. IL-17 stimulates astrocytes to produce CXCL1, which recruits neutrophils into the CNS and thus enhances inflammation and damage (Gelderblom et al., 2012). Furthermore, in vitro studies have shown that astrocytes (Meeuwsen et al., 2003) and microglia (Waisman et al., 2015) release IL-17 in response to pro-inflammatory stimuli and cerebral ischemia reperfusion, respectively.

On the other hand, IL-17 promotes the expression of neuroprotective molecules such as brain-derived neurotrophic factors (BDNF), glia-derived neurotrophic factors (GDNF), and nerve growth factors (NGF), suggesting that IL-17 may play a role in damage reduction (Kawanokuchi et al., 2008). Recombinant mouse IL-17 has recently been...
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Enhanced inflammation within the cerebellum following CisPt exposure was evident in the significant histological alterations in the cerebellum of pups that was discussed earlier.

Conclusion and recommendation: This study suggests that inflammatory cytokines; IL-6 and IL-17 may have a possible role in the cerebellar neurotoxicity following infantile CisPt-exposure. Ancillary studies are needed to determine whether anti-IL-17 and anti-IL-6 therapies will be effective in limiting the harmful inflammation-related effects within the cerebellum or not.

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REFERENCES:


43. Sengupta, P. (2013). Environmental and occupational exposure of metals and their role in male reproductive functions. Toxicology and Chemical Environmental Exposure of metals and their role in male reproductive functions. Toxicology and Chemical Environmental Exposure of metals and their role in male reproductive functions. Toxicology and Chemical Environmental Exposure of metals and their role in male reproductive functions. Toxicology and Chemical Environmental Exposure of metals and their role in male reproductive functions. Toxicology and Chemical Environmental Exposure of metals and their role in male reproductive functions. Toxicology and Chemical Environmental Exposure of metals and their role in male reproductive functions. Toxicology and Chemical Environmental Exposure of metals and their role in male reproductive functions. Toxicology and Chemical Environmental Exposure of metals and their role in male reproductive functions. Toxicology and Chemical Environmental Exposure of metals and their role in male reproductive functions. Toxicology and Chemical Environmental Exposure of metals and their role in male reproductive functions. Toxicology and Chemical Environmental Exposure of metals and their role in male reproductive functions. Toxicology and Chemical Environmental Exposure of metals and their role in male reproductive functions. Toxicology and Chemical Environmental Exposure of metals and their role in male reproductive functions. Toxicology and Chemical Environmental Exposure of metals and their role in male reproductive functions. Toxicology and Chemical Environmental Exposure of metals and their role in male reproductive functions. Toxicology and Chemical Environmental Exposure of metals and their role in male reproductive functions. Toxicology and Chemical Environmental Exposure of metals and their role in male reproductive functions. Toxicology and Chemical Environmental Exposure of metals and their role in male reproductive functions. Toxicology and Chemical Environmental Exposure of metals and their role in male reproductive functions. Toxicology and Chemical Environmental Exposure of metals and their role in male reproductive functions. Toxicology and Chemical Environmental Exposure of metals and their role in male reproductive functions. Toxicology and Chemical Environmental Exposure of metals and their role in male reproductive functions. Toxicology and Chemical Environmental Exposure of metals and their role in male reproductive functions. Toxicology and Chemical Environmental Exposure of metals and their role in male reproductive functions. Toxicology and Chemical Environmental Exposure of metals and their role in male reproductive functions. Toxicology and Chemical Environmental Exposure of metals and their role in male reproductive functions. Toxicology and Chemical Environmental Exposure of metals and their role in male reproductive functions. Toxicology and Chemical Environmental Exposure of metals and their role in male reproductive functions. Toxicology and Chemical Environmental